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Biofertilizers as a sustainable strategy of phosphorus efficiency in agricultural soils

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Biofertilizers as a sustainable strategy of phosphorus efficiency in agricultural soils

This dissertation was performed at the Ecology lab at the Centre for Ecology, Evolution and Environmental Changes (cE3c) in a collaborative partnership with Soilvitae - Teclabs under the supervision of Prof. Dr. Cristina Cruz and Dr. Patrícia Correia in the scope of the Master in Ecology and Environmental Management of the Faculty of Sciences of the University of Lisbon.

Resumo

O fósforo (P) é um macronutriente essencial para todos os organismos vivos, encontrando-se presente no solo em concentrações que variam entre 400 – 1200 mg kg^{-1}. É um recurso essencial para a produção de alimentos, contudo a sua disponibilidade é limitada uma vez que as plantas só conseguem absorver P na forma solúvel a qual representa apenas 20% do P presente no solo.

A maioria dos solos agrícolas contém grandes reservas de P, uma parte considerável dos quais se acumulou como consequência de aplicações regulares de fertilizantes fosfatados, provenientes da rocha fosforite. A fosforite é uma fonte de P não renovável, e o seu uso intensivo na agricultura tem levado à depleção deste recurso natural. Consequentemente, a União Europeia (UE) tem desafiado a comunidade científica para encontrar soluções para este problema, e tem feito recomendações aos produtores agrícolas para reduzir a adubação em cerca de 33 %.

Os microrganismos desempenham um papel fundamental no ciclo biogeoquímico do P. Entre a grande diversidade de microrganismos do solo, os fungos micorrízicos arbusculares (AMF) e as bactérias solubilizadoras de fósforo (PSB) estão diretamente envolvidos na aquisição de P pela planta. Muitos estudos apontam para os efeitos benéficos e cooperativos da inoculação de AMF e/ou PSB no crescimento de determinadas plantas, assim como para a aquisição de P. No entanto, a maioria dos seus efeitos benéficos é observada em experiências conduzidas tanto *in vitro* como em solo estéril. Um teste mais realista do seu potencial seria avaliar se a adição de inóculos compostos por AMF e/ou PSB a um solo agrícola iria proporcionar um benefício no crescimento da planta. Tais testes são importantes porque permitem avaliar o efeito de AMF e PSB num solo com uma comunidade microbiana pré-existente e em condições reais de produção agrícola. Estes microorganismos, se demonstrados os seus efeitos benéficos em solo agrícula, poderiam ser estudados com mais detalhe com vista a serem usados como biofertilizante.

Um biofertilizante é definido como "uma substância que contém microrganismos vivos que, quando aplicados a sementes, superfícies de plantas ou solo, colonizam a rizosfera ou o interior da planta e promovem o crescimento aumentando a oferta ou a disponibilidade de nutrientes primários para a planta hospedeira".

A cultura de cereais é uma das principais causas dos impactos antropogénicos no ciclo biogeoquímico do P, pela demanda contínua de fertilizantes fosfatados e pela remoção sucessiva de P dos campos, associada à recolha dos produtos agrícolas. Há, portanto, necessidade de melhorar a eficiência do uso do P nos sistemas agrícolas, a fim de salvaguardar as reservas deste e a nossa segurança alimentar. Isto poderia ser alcançado aumentando a absorção de P do solo pela planta (eficiência de aquisição de P; PAE) e/ou aumentando a produtividade por unidade de P na parte aérea da planta (eficiência de utilização interna de P; PUE).

O principal objetivo deste estudo foi testar uma estratégia de gestão alternativa às práticas atuais de fertilização agrícola, com base na aplicação de biofertilizantes que operam em várias etapas do ciclo do P, a fim de reduzir a aplicação de fertilizantes fosfatados e aumentar a eficiência de P pela planta. A nossa hipótese defende que a aplicação de biofertilizantes adequados pode promover a eficiência das plantas, aumentando a produtividade e a qualidade dos alimentos, e reduzindo a aplicação de grandes quantidades de fertilizantes fosfatados.

Especificamente as seguintes questões foram abordadas para avaliar a estratégia de gestão agrícola proposta: a) Será que inoculantes microbianos aumentam a eficiência do P em plantas de milho num sistema agrícola? b) Será que os inoculantes microbianos promovem a produtividade do milho e a

qualidade do grão? c) Será que inoculantes microbianos promovem o crescimento das plantas e a eficiência do uso do P pela planta sob um regime de fertilização reduzida (redução de 33% de P)? E esse efeito depende da riqueza específica e identidade das espécies microbianas? Para responder a estas questões, dois sistemas experimentais foram estabelecidos.

Num sistema agrícola intensivo de milho, três inoculantes microbianos foram testados (AMF - *Rhizoglomus irregulare* -, PSB - *Pseudomonas sp.* - e AMF+PSB). Foi mostrado que: i) PSB e AMF+PSB aumentaram a produtividade das plantas, especificamente a forragem verde; ii) PSB aumentou PAE, através do aumento do conteúdo de P na planta; iii) AMF+PSB aumentou PUE; iv) todos os inoculantes afetaram a alocação de P na planta, resultando numa menor concentração de P no grão.

Embora o inoculante PSB não seja o mais eficiente na promoção da produção de biomassa vegetal, é de facto o mais eficiente no aumento do teor de P na planta e, portanto do valor nutricional da forragem verde do milho, aumentando o teor de P na palha (folhas e colmo) e reduzindo a concentração de P no grão, o que poderia ser extremamente útil em sistemas de produção onde a disponibilidade de P é realmente baixa.

Tendo em consideração o panorama Europeu, onde os solos contêm mais P do que o recomendado, mesmo que indisponível para as plantas, os resultados obtidos com o consórcio AMF+ PSB são mais relevantes. AMF+PSB foi o inoculante mais eficiente em promover o crescimento das plantas por unidade de P no tecido vegetal. Esta pode ser uma característica importante no aumento do valor nutricional do grão de milho. Este inoculante poderá, no futuro, ser testado como uma forma de reduzir os insumos de P, idealmente em 33%, como recomendado pela UE, para verificar se a produtividade é mantida. Portanto, este consórcio AMF+PSB poderá ser um dos principais contribuintes na produção de recursos alimentares de alto valor, com aumento zero na degradação da terra, reduzindo os impactos ambientais negativos. Este inóculo foi sugerido para ser testado num ensaio de vaso como forma a reduzir a aplicação de P no solo.

Num ensaio de vaso onde a fertilização de P foi reduzida (33% menos que a dose recomendada de fertilizante), testámos distintos consórcios microbianos (uma espécie de AMF - *Rhizoglomus irregulare* - combinada com diferentes espécies de PSB - *Pseudomonas sp.* 1, *Pseudomonas sp.* 2, *Pseudomonas sp.* 3 -) no desempenho da planta e eficiência de P. Esperava-se que um consórcio microbiano com mais espécies fosse mais eficiente em promover o crescimento de plantas e a eficiência de uso de P, obtendo respostas da planta similares àquelas obtidas com 100% de fertilização de P. Foi demonstrado que: i) consórcios com a mesma riqueza específica, que incluem uma espécie de AMF combinada com uma única espécie de PSB (1, 2 ou 3), apresentaram plantas com crescimento e características fisiológicas e nutricionais (P) semelhantes, porém estas diferenças não são significativas quando comparadas com plantas dos controlos não inoculados; ii) o aumento da riqueza específica em consórcios pela combinação de AMF com duas espécies de PSB mostrou efeitos inconsistentes no desempenho da planta, dependendo do consórcio. O consórcio AMF+PSB(1+3) aumentou o crescimento das plantas e a nutrição em P, enquanto o AMF+PSB (1+2) não promoveu essas características quando comparado às plantas controlo. iii) as plantas tratadas com o consórcio com mais espécies, resultado da combinação de AMF e três espécies de bactérias (AMF+PSB(1+2+3)) apresentaram o melhor desempenho, revelando morfologia, nutrição em P e eficiência da aquisição do P semelhante às plantas de um controlo não inoculado com 100% de fertilização de P. Portanto, a maior eficiência do uso do P para solos agrícolas foi alcançada com consórcio com mais espécies.

Este estudo mostrou que um consórcio microbiano contendo AMF+PSB pode melhorar o desempenho da planta e a eficiência do uso do P em solos agrícolas. Os nossos resultados indicam também que, dependendo da combinação de bactérias, existe um potencial para cooperação entre AMF

e PSB para promover a aquisição de P, porém cada consórcio afetou a eficiência do uso do P de maneiras distintas. Os nossos resultados chamam a atenção para a necessidade de ter em consideração as relações entre os componentes do consórcio e entre estas e o hospedeiro no efeito do biofertilizante na cultura, o que pode explicar as inconsistências reportadas para o efeito do biofertilizante.

A política agrícola comum (PAC) na UE poderá promover o uso de biofertilizantes como uma ferramenta para corrigir a contribuição da agricultura para o desequilíbrio do ciclo biogeoquimico do P. Deste modo os países da UE poderiam tornar-se menos dependentes da disponibilidade de reservas de fosforite, bem como caminhar para uma intensificação sustentável da agricultura. O uso de microrganismos rizosféricos como AMF e PSB em consórcios multifuncionais polimicrobianos pode ser o despertar para uma intensificação agrícola mais sustentável baseada numa estratégia sustentável do uso do P em solos agrícolas.

Palavras-chave: Consórcios rizosféricos; Bactérias solubilizadoras de fósforo; Fungos micorrízicos arbusculares; Fósforo no solo; Cooperação.

Abstract

Phosphorus (P) is one of the major limiting factors for crop growth. Even though P is a non-renewable resource and its reserves are drastically decreasing, it is continuously applied in the form of mineral fertilizers in intensive agricultural systems, to increase crop production. The consequences of both its overuse and depletion have led to inestimable environmental damages and has brought insecurity to economies.

Microorganisms play a key role in the biogeochemical cycle of P. Among the great diversity of soil microorganisms, arbuscular mycorrhizal fungi (AMF) and phosphorus solubilizing bacteria (PSB) are directly involved in plant P acquisition. Many studies point to the beneficial effects of AMF and / or PSB inoculation on the growth of certain plants, as well as on P. If the beneficial effects of these microorganisms are proven in agricultural soil, they could be further studied with the aim of developing a viable biofertilizer.

Biofertilizer is defined as "a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant".

Grain crops are one of the key drivers of the current global P cycle through its continued demand for P fertilizer, and the successive removal of P from fields in the harvested grain. Therefore, there is a need to improve P efficiency in agricultural systems in order to safeguard P and food security. This could be accomplished by increasing P uptake from soil (P-acquisition efficiency; PAE) and/or by enhancing productivity per unit of P in the shoot of the plant (internal P utilization efficiency; PUE).

The main goal of this study was to test an alternative management strategy to the current agricultural fertilization practices, based on the application of biofertilizers that operate at various stages of the P cycle in order to reduce P fertilizer application and increase plant P use efficiency. It has been hypothesized that application of suitable biofertilizers promote plant P use efficiency, increasing plant productivity and food quality, and reducing the input of large amounts of P fertilizers. To achieve this, two experimental systems were established.

In an intensive maize agricultural field, three different microbial inoculants were tested: Arbuscular mycorrhizal fungi (AMF - *Rhizoglomus irregulare* -), Phosphate solubilizing bacteria (PSB - *Pseudomonas sp.* 1, *Pseudomonas sp.* 2, *Pseudomonas sp.* 3 -) and AMF+PSB. It was shown that: i) PSB and AMF+PSB enhanced plant productivity, specifically green forage; ii) PSB increased PAE, by increasing P content; iii) AMF+PSB increased PUE; iv) all inoculants affected P allocation within the plant, resulting in lower P concentration in the grain.

When looking at the European panorama, where soils contain more P than recommended, even if unavailable to plants, the results obtained with the consortium inoculum (AMF+PSB) are more relevant. This inoculum was further tested on a pot experiment as a way to reduce P inputs.

In a reduced P fertilization pot assay (33 % less than the recommended fertilizer dose), we tested distinct microbial consortia (one AMF species combined with different species of PSB) on plant performance and P use efficiency. It was expected that a more diverse bacterial consortium would be more efficient at promoting plant growth and P use efficiency, attaining plant responses similar to those obtained with 100 % P fertilization. It was shown that: i) consortia with similar species richness, that includes one AMF species combined with one single species of PSB (1, 2 or 3), showed similar plant growth, physiological and P nutrition characteristics among them, however not significantly different from the uninoculated controls; ii) Increasing species richness in consortia by combining AMF with two

species of PSB showed inconsistent effects in plant performance depending on the consortium. One of the consortia (AMF+PSB(1+3)) increased plant growth and P nutrition, while the other (AMF+PSB(1+2)) did not promote these traits when compared to the control plants. iii) plants treated with the most diverse consortium, resulted of combining AMF and three species of bacteria (AMF+PSB(1+2+3)) had the best performance, revealing growth morphology, P nutrition, P acquisition efficiency similar to an uninoculated control with 100 % P fertilization. Therefore, the highest P efficiency for agricultural soils was achieved with the most diverse consortium.

This study showed that a microbial consortium containing AMF+PSB can improve plant performance and P use efficiency in agricultural soils. Our results also indicate that, depending on the bacterial combination, there is a potential for cooperation between AMF and PSB to promote P acquisition, but each consortium affected the P use efficiency in different ways. Our results point out the need to take into account the relationships between the components of the consortium and between the host crop and the biofertilizer, which may explain the inconsistencies reported for the biofertilizer effect.

Overall, the use of rhizospheric microorganisms such as AMF and PSB in polymicrobial multifunctional consortia may be the wake-up call for a more sustainable agricultural intensification based on a sustainable P-use strategy in agricultural soils.

Keywords: Rhizosphere consortia; Phosphorus solubilizing bacteria; Arbuscular mycorrhizal fungi; Phosphorus in the soil; Cooperation

Contributions

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ACC	1-Aminocyclopropane-1-carboxylic acid
Al	Aluminium
AMF	Arbuscular mycorrhizal fungi
AMF+PSB	Arbuscular mycorrhizal fungi + Phosphate solubilizing bacteria
B	Boro
Ca	Calcium
$Ca(NO_3)_2$	Calcium Nitrate
CaO	Calcium oxide
CAP	Common agricultural policy
$Ca_3(P_2O_5)$,	Rock phosphate
Cl	Chloride
CTR2	Carter Index
Cu	Copper
$CuSO_4$	Copper sulfate
Dw	Dry weight
ER	Method of Egnér-Riehn
EU	European Union
Fe	Iron
FeNaEDTA	Ferric-Sodium Salt
Fw	Fresh weight
H_3BO_3	Boric acid
$H_2PO_4^-$	Dihydrogen phosphate ion
HPO_4^{2-}	Mono hydrogen phosphate
IAA	IAA – indole-3-acetic acid
K^+	Potassium
KCl	Potassium chloride
KNO_3	Potassium nitrate
K_2O	Potassium oxide
LC	Leaf chlorophyll
Mg	Magnesium
MgO	Magnesium oxide
$MgSO_4$	Magnesium sulfate

Mn	Manganese
$MnSO_4$	Manganese sulfate
Mo	Molybdenum
N	Nitrogen
NDVI	Normalised Difference Vegetation Index
NH_2CONH_2	Urea
$(NH_4)_6Mo_7O_{24}$	Ammonium molybdate tetrahydrate
NHO_4^+	Ammorium
NO_3^-	Nitrate
P	Phosphorus
PAE	Phosphorus acquisition efficiency
PER	Phosphorus efficiency ratio
PGPR	Plant growth-promoting rhizobacteria
Pi	Inorganic phosphorus
P_2O_5	Phosphorus pentoxide
PPUE	Physiological phosphorus use efficiency
PRI	Photochemical Reflectance Index
PSB	Phosphate solubilizing bacteria
PUE	Phosphorus utilization efficiency
PUTE	Phosphorus utilization efficiency
RP	Rock Phosphate
S	Sulfur
SD	Standard deviation
WI	Water Index
Zn	Zinc
$ZnSO_4$	Zinc sulfate

Chapter 1 - General introduction

Feeding the ever-growing human population has been possible mainly due to the use of mineral fertilizers in agriculture. Today, their use has turned into a routine agronomic practice (Childers et al. 2011).

Phosphorus (P) is an essential macronutrient required for plant nutrition and is found in the soil at concentrations ranging between 400 – 1200 $mg.kg^{-1}$ (Cordell and White 2013).

Plants absorb P dissolved in the soil solution in the form of primary orthophosphate, $H_2PO_4^-$ or secondary orthophosphate HPO_4^{2-} (Quelhas dos Santos 1996; Owen et al. 2015). P plays a crucial role in plant energy reactions (energy transfer and storage), photosynthesis and oxidative processes (e.g. respiration). It is a structural component of several biochemical elements of the plant such as phosphoproteins, phospholipids and phytin. Furthermore, it is also an important part of the structure of cell membranes and both DNA and RNA. P deficiency reduces flowering, ripening and the development of both fruits and seeds, resulting in large production losses (Quelhas dos Santos 1996; Varennes 2003).

About 80 % of the inorganic phosphorus (Pi) applied to agricultural fields becomes rapidly immobilized in the soil and consequently cannot be directly acquired by plants because of adsorption, precipitation, or conversion into organic forms (Holford 1997). Furthermore, soil P availability depends on a wide variety of factors such as soil temperature, chemical nature and concentration of the soil organic matter, metals (aluminium, iron and calcium) concentration, competing anions, ionic strength, soil pH, root exudates (Quelhas dos Santos 1996; Varennes 2003) and soil microorganisms (Varennes 2003; Richardson et al. 2011).

1.1. Phosphorus scarcity: What is the big deal?

Rock phosphate (RP) is the world's high-quality source of P and is formed by fossil sedimentary and igneous deposits. It is, however, considered a non-renewable resource, as its formation requires hundreds of millions of years (Cordell and White 2013; Reijnders 2014). Around 90 % of the RP extracted globally is used for food production (Cordell et al. 2009) and, because of agricultural intensification, its demand has been increasing drastically. Consequently, RP reserves are quickly diminishing due to their over exploration, which can lead to price surges in the future (Cordell and White 2011).

Morocco, China and the USA detain more than 85 % of the known RP reserves (Cordell et al. 2009; Cooper et al. 2011). This concentration of P resources is a potential source of tension among countries that depend entirely on imports of RP (Cooper et al. 2011). There is a growing concern that prices may increase dramatically once again, as so happened in 2008, when they reached a peak increase of 800 % relatively to the average price at the time (Cordell and White 2011; Reijnders 2014).

Right now, we are facing a global P system paradox. On one hand, P access is being progressively limited and is, consequently, bringing insecurity to economies. On the other hand, the application of P fertilizers in agricultural areas, to increase crop productivity, keeps rising (Shepherd et al. 2016). Also, the continuous excessive usage of P fertilizers is causing inestimable environmental damage (Tiessen 2008; Childers et al. 2011; Reijnders 2014). The future

consequences of global P scarcity for the world's food supply and food security may be devastating. Even though there is no substitute for P, actions could be taken to improve the efficiency of its use and safeguard this resource. Governments, ONGs, institutions and scientific communities are organising efforts to find sustainable P measures to operate at the various stages of the P cycle, in order to close its conceptual biogeochemical cycle (Cordell and White 2013). These measures can encompass the reconsideration of agriculture management systems, diversification of the P sources used in agriculture, reconsideration of diets based on P-efficient foods, P recycling and increasing P use efficiency (Cordell and White 2013; Reijnders 2014).

1.2. Microbes: The farm's helpers

Vessey (2003) defines a biofertilizer as "a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant". With the ever-increasing demand for more environmentally friendly agricultural practices without significant decreases in productivity, this class of products spurred the interest of being applied alongside mineral fertilizers, which constitute a way to improve soil quality by increasing microbial biodiversity, unlocking the immobilized nutrients and consequently increasing their bioavailability (Bhardwaj et al. 2014; Owen et al. 2015). Among these products those based on mycorrhizae and/or phosphate solubilizing bacteria (PSB) are of special interest due to their well-known functional relationship with plants as well as synergistic and antagonistic interactions with other soil microorganism which contribute to the sustainability of soil fertility (Bhardwaj et al. 2014; Nadeem et al. 2014; Owen et al. 2015).

Gerdemann (1970) defined mycorrhizae as "a mutualistic association between symbiotic soil-borne fungi and plants". These mycorrhizae are able to improve plant growth through the facilitation in acquiring different nutrients (Gerdemann 1970; Bolan 1991; Smith and Read 2008). Arbuscular mycorrhizal fungi (AMF) are partially responsible for the increase of soil P bioavailability from sources not directly available to plants (Smith and Read 2008). The AMF hyphae are able to reach a bigger soil volume than that explored by a plant's roots alone and, consequently, can increase the surface area for absorption of phosphate ions and decrease its diffusion distance. P movement into the roots is accelerate by AMF hyphae due to their high affinity for phosphate ions and by lowering the concentration threshold required for its absorption. AMF hyphae can overcome the nutrient depletion zone formed alongside the root surface due to a faster nutrient uptake relative to nutrient diffusion in the soil solution (Bolan 1991; Smith and Read 2008; Jansa et al. 2013). AMF from the genus *Funneliformis*, *Glomus*, *Rhizoglomus*, *Scutellospora* and *Claroideoglomus* have been vastly studied for their benefits in plant growth and P acquisition (Smith and Read 2008; Owen et al. 2015). These are characterized by the formation of unique structures inside plant roots, such as arbuscules and vesicles (Figure 1.1).

PSB are free-living soil bacteria present in most soils and constitute 1 – 50 % of the soil microbial population (Vessey 2003). Evidence of the natural occurrence of P solubilizing rhizospheric microorganisms dates back to 1903 (Khan et al. 2009b). PSB enhance P availability to plants through the solubilization and mineralization of inorganic and organic soil P forms, respectively (Rodríguez and Fraga 1999; Khan et al. 2009b). The solubilization of P in the rhizosphere is the most common mechanism of increasing P availability to the host plant from inorganic P sources (Khan et al. 2009a; Richardson and Simpson 2011), and is based on the secretion of organic compounds which acidify the medium and consequently increase the

availability of soluble P by PSB, other microorganism and plant (Rodríguez and Fraga 1999; Khan et al. 2009a). Mineralization of organic P occurs mostly via the release and action of acid and alkaline phosphatases (Rodríguez and Fraga 1999; Richardson et al. 2011). The mineralization of organic P plays a key role in the P cycle of an agricultural system, when under low fertilizer input, since organic P may constitute 4 – 90 % of the total soil P (Khan et al. 2009b). PSB also have important roles in plant growth and development by producing plant growth promoting substances, such as phytohormones, or by producing antibiotics, siderophores and cyanide (important for biocontrol). The use of P solubilizing microorganisms can increase crop yields by up to 70 %. Bacteria species of *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* have been described as effective in P solubilization (Rodríguez and Fraga 1999).

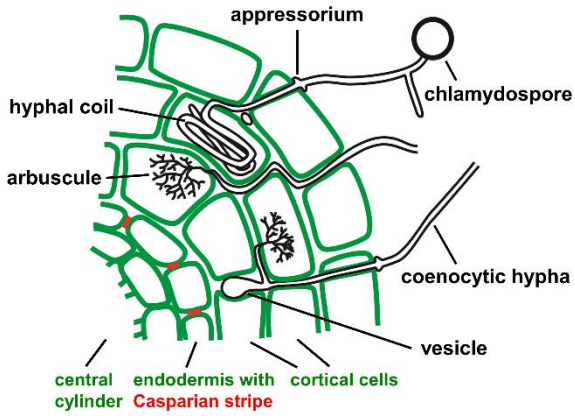

Figure 1.1 Arbuscular mycorrhiza scheme. Symbiotic association between an AMF and the roots of a host vascular plant. The AMF penetrates the cortical cells of the root and forms arbuscules and vesicles. Author: Piepenbring, 2015.

The combination between AMF and PSB is known to be highly relevant for plant growth and nutrition (Toro et al. 1997; Owen et al. 2015; Ordoñez et al. 2016; Zhang et al. 2016). It can improve P uptake by plants because PSB can potentially raise the amount of soluble P forms in the soil that may be absorbed and transported to the plant by AMF hyphae (Owen et al. 2015; Zhang et al. 2016). PSB and AMF establish a chemical crosstalk that may influence the chemical, physical and biological environment of the rhizosphere (Zhang et al. 2016) in a beneficial way for the plant that mediates the process through the production of root exudates.

Although much has been done in laboratory and greenhouse settings, the interaction between AMF and PSB is poorly understood when applied to agriculture, one of the reasons being the fact that most experiments are performed in sterile conditions (e.g. sterile soil). The outcomes of the inoculation of rizhospheric microorganisms in field trials has had variable success, because most past trials only used a strain of microorganisms in the field, as well as the majority of the biofertilizers available in the market (Owen et al. 2015; Rodriguez and Sanders 2015; Ordoñez et al. 2016).

1.3. Objectives and strategy

To solve the problematic of global P resources depletion and high P demanding agricultural practices, novel strategies regarding P use efficiency are needed, as has been encouraged by the European Union.

The main goal of this study was to test an alternative management strategy to the current agricultural fertilization practices, based on the application of biofertilizers that operate at various stages of the P cycle in order to reduce P fertilizer application and increase plant P use efficiency. It has been hypothesized that application of suitable biofertilizers promote plant P use efficiency, increasing plant productivity and food quality, and reducing the input of large amounts of P fertilizers.

We chose *Zea mays* L. as the host plant since it is: i) a fast-growing cereal crop with great economic and nutritional importance worldwide (Ranum et al. 2014); ii) has a particularly high nutrient requirement, specially P, to meet genetic potential for growth and yield (Nadeem et al. 2011); iii) highly dependent on AMF (Aquino et al. 2015) and several works report beneficial and cooperative effects of its culture in association with PSB (Hameeda et al. 2008).

Specifically, the following questions were addressed to evaluate this proposed agricultural management strategy:

1. Do microbial inoculants increase P use efficiency in maize plants in an agricultural system?

2. Do microbial inoculants promote maize productivity and grain quality?

3. Do microbial inoculants promote plant growth and plant P use efficiency under a reduced fertilization regime (33 % P reduction)? Does this effect depend on the richness and specificity of the microbial species?

To answer these questions, two experimental systems were established:

The first was implemented in an intensive maize agricultural field where three different microbial inoculants (AMF, PSB and AMF+PSB) were tested. It was expected that the microbial consortium (AMF+PSB) would induce higher P use efficiency and higher P extraction from the soil by the host plant, due to the complementary functions of PSB and AMF in relation to phosphate transformations and uptake.

The second experiment was implemented as a reduced P fertilization pot assay (33 % less than the recommended fertilizer dose), testing distinct microbial consortia (one AMF species combined with different species of PSB) on plant performance and P use efficiency. It was expected that a more diverse bacterial consortium would be more efficient at promoting plant growth and P use efficiency, attaining plant responses similar to those obtained with 100 % P fertilization.

1.4. Dissertation Structure

This work consists of four chapters and is structured as follows:

Chapter 1- General introduction: Establishes the state of the art and reviews the problematic of P, the importance of P in agriculture, defines biofertilizer and the role of microorganisms in agriculture. Lastly, the main goal and strategy of this work are outlined.

Chapter 2 - Biofertilizers and phosphorus efficiency in farm: Discusses the effect of three microbial inoculants, which can potentially become biofertilizers, on the phosphorus use efficiency of maize plants of an intensive agricultural system, under field conditions.

Chapter 3 - The relevance of bacteria consortia in phosphorus efficiency: Assesses, under greenhouse conditions (pot experiment), if increasing species richness in a microbial consortium enhances its efficacy when P fertilization is reduced by 33 %.

Chapter 4 - General discussion: Discusses the purpose of this work, relates our findings with our hypothesis and with those obtained by other researchers. Also, states the main conclusions of our findings, their significance with regard to the actual inefficiency of P in agricultural soils and some practical applications and future studies that can be developed.

Chapter 2 - Biofertilizers and phosphorus efficiency in farm

2.1. Introduction

Enhanced P efficiency in agricultural soils can be achieved if plant production increases at a given rate of P fertilizer application, or if production remains stable with lower P-inputs (Richardson et al. 2009b; Rose et al. 2013). This could be accomplished by increasing P uptake from soil (P-acquisition efficiency; PAE) and/or by enhancing internal P utilization efficiency (PUE) (Wang et al. 2010; Rose and Wissuwa 2012; Veneklaas et al. 2012). In both high- and low-input agricultural systems a combination between these two strategies (Rose and Wissuwa 2012; Heuer et al. 2017) is desirable. In general, most of the research has been focused on improving P use efficiency through breeding programs or in studying mutant plants, even though some studies did show an increase in P use efficiency. The long-term sustainability of these strategies, to safeguard soil quality through preventing erosion and nutrient leaching or even avoiding large losses of biodiversity, has not been thoroughly explored. The genetic and molecular mechanisms conferring plants improved PAE have been widely reviewed and some strategies to increase PAE include molecular plant breeding, deployment of transgenic plants (genetic engineering) and use of agricultural practices that enhance plant growth through the inoculation of plant growth-promoting rhizobacteria (PGPR) and mycorrhizae (Ramaekers et al. 2010). Contrariwise, far less is known on how to improve PUE (Rose and Wissuwa 2012). PUE is poorly understood and there are several factors that contribute to it, such as a lack of a single definition (e.g., some authors refer to PUE as grain yield per unit of P fertilizer applied, and others as plant biomass per P present in specific tissues) and inconsistent use of its acronyms. Parameters such as P-efficiency ratio (PER), P utilization efficiency (PUTE), P internal utilization efficiency (PUTIL), or even physiological P use efficiency (PPUE) are used to assess P utilization efficiency.

In this work we will use the definition of PUE in a plant physiological/internal perspective as the biomass produced (grain, stover or shoot biomass) per unit of P present in the shoot (Rose et al. 2011; Rose and Wissuwa 2012; Veneklaas et al. 2012; van de Wiel et al. 2016). However, this definition of PUE does not account for the balance between P inputs (fertilizer addition) and P outputs (P exported from the system at crop harvest), which disruption is the cause of significant environmental problems (Tiessen 2008; Childers et al. 2011). In an ideal agricultural system, P outputs should be balanced by P inputs and the biomass production per unit P uptake should be high (higher PUE) (Veneklaas et al. 2012). To increase P eficiency in agricultural soils we propose the use of rhizospheric microorganisms in combination with fertilizers, as a sustainable strategy.

Microorganisms play a key role in the natural cycle of P. This cycle occurs through cyclic oxidation and reduction of P compounds. Among the wide diversity of soil microorganisms, arbuscular mycorrhizal fungi (AMF) and phosphorus solubilizing bacteria (PSB) are directly involved in P turnover and plant P acquisition (Zhang et al. 2014). Plants naturally interact with soil microbial communities, including existing populations of both AMF and PSB (Owen et al. 2015). Many studies point out the beneficial and cooperative effects of AMF (Smith and Read 2008) and/or PSB inoculation (Zhang et al. 2014, 2016; Ordoñez et al. 2016) for plant growth, P uptake and as an acting biological tool for eco-restoration (Khan 2006). However, most of its

beneficial effects are observed in experiments conducted in microcosm experiments or in sterile soil (Rodriguez and Sanders 2015). A more realistic test of their potential is whether adding AMF and/or PSB inoculum to an agricultural field will give a growth benefit to the plant. Such experiments are important because they test the effect of the AMF and PSB under a soil environment and existing microbial community.

The combined use of bio- and synthetic fertilizers may be the fine-tuning necessary to increase resource use efficiency of European Union (EU) agricultural systems where intensification is already very high. The connection between increased inputs and increased resource use efficiency goes against the Law of Diminishing Returns which, when applied to agriculture postulates that the increase in plant growth promoted by a fertilizer is proportional to the amount of fertilizer applied only until a certain limit, having less and less effect the more it is applied past that limit. However, biofertilizers can control nutrient bioavailability in the soil by holding more complex interactions with the soil structure and, in agreement with the Law of Diminishing Returns, are more efficient at lower than at higher nutrient concentrations (Warton et al. 2015; Weltin et al. 2018). Therefore, the adequate use of biofertilizers may provide the win-win strategy necessary for sustainable intensification of agriculture in EU and beyond, allowing the production of more crop with less input of fertilizer.

Cereal crops are very P demanding, fertilizer doses ranging from 10 to 250 kg ha^{-1} are commonly used in Europe and in Portugal (Varennes 2003; Amery, F., Schoumans 2014). Depending on the farm management the amount of P used by the crop may range between 10 – 50 % of the recommended dose of P fertilizer (Varennes 2003; MacDonald et al. 2011; Amery, F., Schoumans 2014), which implies that the agro-systems are subjected to either a continuous P enrichment or significant losses to the surrounding ecosystem (Varennes 2003; MacDonald et al. 2011). In cereals, it is estimated that 60 – 85 % of the total P acquired by the plants is allocated to the grains in the form of phytate, which is essential for seed germination and seedling vigour. However, the concentration of P in the seeds can be reduced without compromising plant growth or vigour (Rose et al. 2013; Yamaji et al. 2017). A major concern is that phytate cannot be digested by humans and other monogastric animals, which results in large quantities of P in animal excrements. Globally, this is one of the main contributions of organic waste to the eutrophication of rivers and lakes (Cordell and White 2013). Moreover, given the non-renewable nature of P reserves and the speed at which they are now exploited for fertilizer production, it is critical to increase P use efficiency in agricultural systems in order to safeguard P and food security (Cordell and White 2013; Rose et al. 2013).

The goal of this study was to evaluate, under field conditions, the effect of three inoculants (AMF, PSB and AMF+PSB) with potential to become biofertilizers, on the P efficiency of an intensive agricultural system.

We hypothesized that the inoculants would: i) promote plant P uptake from soil and, consequently PAE; and ii) enhance internal PUE by producing more biomass per unit of P uptaken, which will also reduce the concentration of phytate in the grain.

2.2. Material and methods

2.2.1. Growth conditions

This study, with a duration of 110 days, was conducted in a farm, located in Lourinhã, Lisbon, Portugal (39° 16' 32.3'' N 9° 17' 27.4'' W), from early June to late September 2016. Daily mean air temperature was 24 ºC and ranged between 16 - 32 ºC, relative humidity ranged between 53.2 - 67.7 %, according to Instituto Português do Mar e da Atmosfera (IPMA). These values represents the average obtained from site equidistant meteorological stations (IPMA 2016) (Appendix 1).

Field soil had a coarse sand texture, 0.8 % of organic matter, pH (H_2O) 6.3 and extractable P (Egnér-Riehm method) of 442 ppm (analysis performed by Laboratório de Solos e Plantas, UTAD, Portugal, 2016).

Zea mays L., cultivar *Sincere* (*Syngenta*) seeds were hand sowed on the 4th of June 2016. Plants were grown 0.75 m apart between rows and with 0.20 m spacing along the row, the equivalent of ~ 67 000 plants ha^{-1} (Ormonde da Silva 2012). Before sowing, a basal fertilization of 16 kg NO_3^-, 48 kg NHO_4^+, 96 kg P_2O_5 and 96 kg K_2O ha^{-1} was applied. This was followed by a top-dressing fertilization, 6 weeks after sowing, of 60 kg NHO_4^+, 180 kg NH_2CONH_2, 96 kg P_2O_5 and 120 kg K_2O ha^{-1} (according to the recommendations of ADP Fertilizantes).

2.2.2. Experimental design

The experiment lay out was set up as a complete randomized block design with a total area of 27 m^2 (3 x 9 m). There were a total of 3 blocks, containing 4 parcels each, comprising 3 inoculants and the control (Figure 2.1). Each parcel had an area of 2.25 m^2. Plants in an area of 5.4 m^2 in both the east and west side of the experimental area were defined as plant guard rows and not used for data collection.

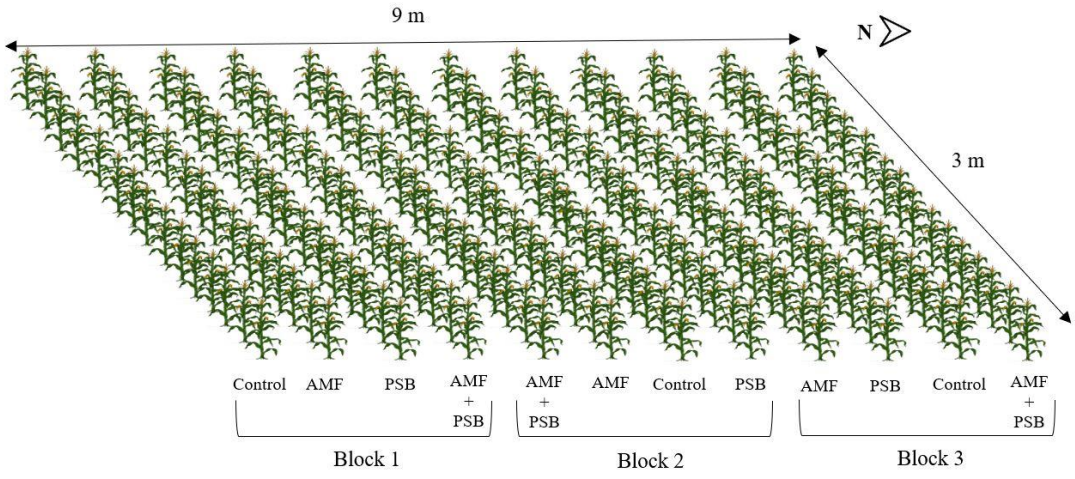

Figure 2.1 Field experimental design. The experimental area (3 x 9 m) was composed of three blocks of four parcels each, comprising three inoculants (AMF, PSB and AMF+PSB) and the control, randomly distributed.

The field trial tested the effect of three inoculants on maize productivity and P use efficiency. The AMF inoculant consisted of propagules and spores of *Rhizoglomus irregulare* (Figure 2.2) from *Symbiom* (*https://www.symbiom.cz/en*). AMF propagules and spores were isolated and counted from a commercial product to estimate the amount of product added to each plot (Appendix 2). At the time of sowing (T_0), AMF inoculant was spread on the soil over the planting furrows corresponding to the treatments with AMF and AMF+PSB. The inoculation corresponded to $2.5x10^6$ AMF spores ha^{-1}, similarly to the recommendations for commercial products trials (Cozzolino et al. 2013).

Figure 2.2 *Rhizoglomus irregulare* spores. Scale 20 µm

PSB inoculant consisted of *Pseudomonas spp.* species 1 (this designation will be useful for the next chapter) and the respective culture medium. We used one *Pseudomonas spp.* species, previously isolated from a Portuguese agricultural soil, which belong to *Soilvitae* collection of PGPR. These bacteria were characterized as PSB because of its capacity to solubilize tri-calcium phosphate and phytate, in *in vitro* conditions.

PSB was inoculated over the area corresponding to PSB and AMF+PSB treatments, in a dose of about 10^{12} CFUs ha^{-1}. A second inoculation of PSB was performed 15 days after sowing (T_{15}), using the same dose as in T_0.

AMF+PSB was a combination of the previous two inoculants using the same dose in a single application. The control did not receive any inoculant.

2.2.3. Sampling and data collection

On the 22th September, plants were manually harvested with the help of loppers. The harvest was carried out when plants reached physiological maturity, and when grains were on the milk to dough phenological phase. Sampling was limited to a central sampling area of 1.35 m^2 per parcel of 16.2 m^2, all border plants were excluded, to avoid heterogeneity between the experimental units, since plants from the borders tend to be more vigorous and more productive than those that grow inside the experimental units, due to the smaller effect of competition between plants and different light exposure (Arruda 1959).

Maize aboveground tissues were classified as plant shoot. The number of tillers per plant was determined and shoots were separated in culm (leaves included) and ear maize (cobs and grain)

and then weighted. Fresh weight (Fw) of plants from each parcel was measure in the field with a field weighing scale.

A sub-sample of 3 plants per parcel (randomly chosen), was taken to oven dried at 65 ºC, until constant weight, which took approximately 3 and 7 days, respectively for ear maize and culm. Then the dry weight (Dw) of shoots (shoot biomass), culm and cobs (stover biomass), and grain (grain biomass) were determined using a precision weighting scale (precision ± 0.01 g, model PGW 3502e digital balance).

From the dry sub-samples, the grain and three leaves from each plant were reduced to powder using a mill of spheres (Retsch MM 2000). Ground samples were used to perform grain and leaf analyses to determine P concentration, using an Optical Emission Spectroscopy after acid digestion (Huang and Schulte 1985) (analysis performed by Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), Murcia, Spain 2016).

Soil samples were collected from bare soil (composite sample, n=5) until a maximum depth of 15 cm. Samples were air-dried and then analysed for chemical and physical properties: extracted P (Egnér-Riehm method), organic matter quantification and pH (H_2O) (analysis performed by Laboratório de Solos e Plantas, UTAD, Portugal, 2016).

2.2.4. Calculations and statistics

The average Fw of culm and ear maize of each parcel and the average shoot Dw of the 3 sub-samples were represented in tonne per hectare (t ha^{-1}) by multiplying the average parcel value of Fw or Dw by the number of plant density used per hectare (~ 67 000 plants). Shoot Fw per hectare was defined as green forage and shoot Dw as the biomass.

The dry matter content of the green forage (%) was assessed by multiplying plant biomass (kg) per 100 and divided by the green forage (kg).

Phosphorus acquisition efficiency (PAE) which represents the amount of P uptaken per plant (Wang et al. 2010; Vandamme et al. 2016) was evaluated through shoot P extraction and P fertilizer recovery efficiency, as follows:

(2.1)

$$P\ extraction = Biomass(Dw\ tissue) \times P\ tissue\ concentration \times Plant\ density$$

(2.2)

$$P\ fertilizer\ recovery\ efficiency = \frac{P\ extraction(shoot)}{P\ applied\ in\ the\ field}$$

P extraction (kg ha^{-1}) reflects the total P content in plant tissues: shoot, stover and grain. This was calculated by combining grain biomass (g) and stover biomass (g) with respective P concentration (g P/100 g plant) for each treatment and was estimated for a hectare by multiplying the result with plant density, as depicted in formula (2.1). We used leaf concentration to estimate stover concentration (Cavaco and Calouro 2006). Shoot P extraction was obtained through the

sum of grain and stover P extraction, while shoot concentration was obtained by dividing shoot P extraction by shoot biomass.

P fertilizer recovery efficiency (kg ha^{-1}) reflects the ability of a plant to acquire nutrients applied to the soil (Baligar et al. 2001) and was calculated by shoot P extraction (kg) divided by the amount of P fertilizer applied (kg ha^{-1}), as depicted in formula (2.2).

Phosphorus Utilization Efficiency (PUE) was evaluated through the amount of biomass (Shoot, Stover or Grain Dw) produced per unit of P present in the shoot (shoot P extraction) (Rose and Wissuwa 2012), as follows:

(2.3)

$$PUE = \frac{Biomass(Dw\ tissue)}{P\ extraction(shoot)}$$

PUE (kg Dw kg P extracted ha^{-1}) was expressed in kg of tissue biomass produced per kg of P present in the shoot in a hectare, formula (2.3).

The effect of the inoculants on maize performance was tested via one-way analysis of variance (ANOVA). Differences among treatment means were determinate by Tukey test ($p \leq 0.05$). The statistical analysis was achieved by analysis of variance that included main effects of treatments, using the software IBM SPSS Statistics version 25. Graphs were developed with GraphPad Prism version 6 (GraphPad Software, San Diego, CA).

2.3. Results

2.3.1. Effect of inoculants on plant productivity

The maize variety tested in this field experiment can be used for forage and grain production, as such crop productivity was assessed based on green forage (Fw) and biomass (Dw) productivity (Figure 2.3 and Figure 2.4).

PSB and AMF+PSB inoculants increased forage productivity relatively to the control plants by 41 % and 48 %, respectively. No significant difference was detected between the AMF treated plants and control plants (ANOVA green forage productivity $F_{3,6}$=9.90, P≤0.01) (Figure 2.3). Inoculants did not significantly affect the Fw partitioning between culm and ears, the two components of the green forage (ANOVA ratio Ear/Culm $F_{3,6}$=0.82, P>0.05).

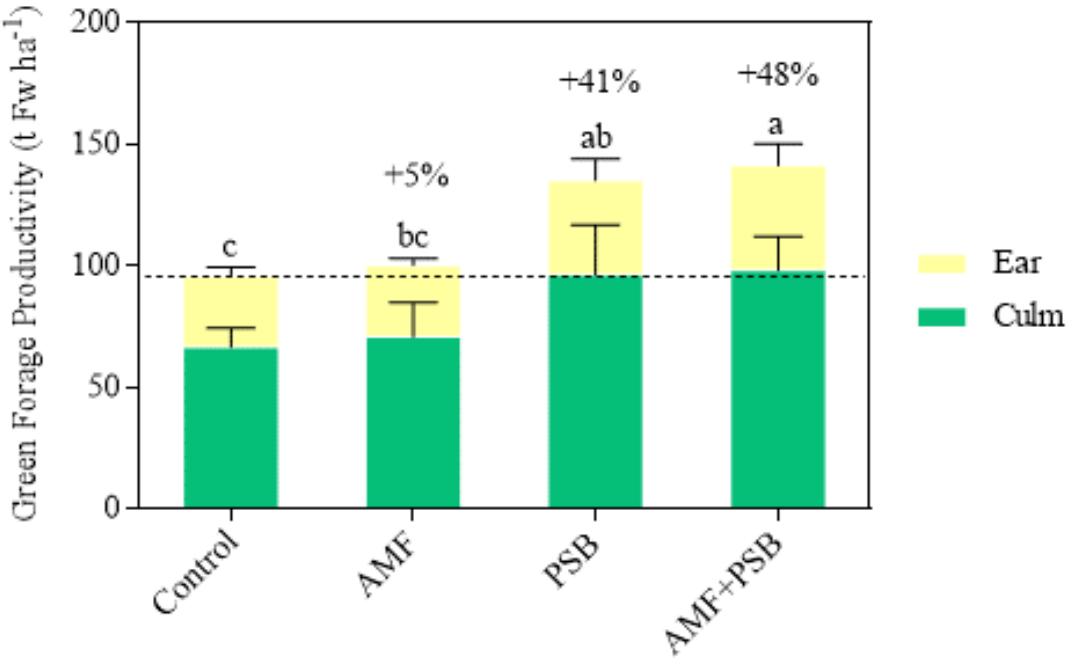

Figure 2.3 Effect of three inoculants (AMF, PSB and AMF+PSB) on maize green forage productivity tested under field conditions. Stacked bars (green and yellow) represent the partition of the average fresh weight of the vegetative (culm) and reproductive (ear) structures. Values shown at the top of the bars refer to the average percent of increment of maize green forage promoted by the respective inoculant when compared to the control plants. Each bar represents the mean of 3 sampling plots ± SD (n=3). Different letters show significance at 5% level (for total green forage productivity), according to Tuckey's HSD test. The dashed line marks the mean value of the fresh weight of maize green forage of the control plants.

The same trend was observed for shoot biomass accumulation as a function of inoculant treatment, with PSB and AMF+PSB inoculants tending to produce bigger plants relatively to the control (increment of 53 % and 65 %, respectively), but no significant differences were found (ANOVA biomass $F_{3,6}$=3.08, P>0.05) (Figure 2.4). Inoculants did not significantly affect the dry weight partitioning between stover and grain, the two main components of shoot biomass (ANOVA ratio Grain/Stover Dw $F_{3,6}$=0.25, P>0.05), not even the Dw of each component, when considered separately (ANOVA Stover Dw $F_{3,6}$=2.77, P>0.05; Grain Dw $F_{3,6}$=3.54, P>0.05).

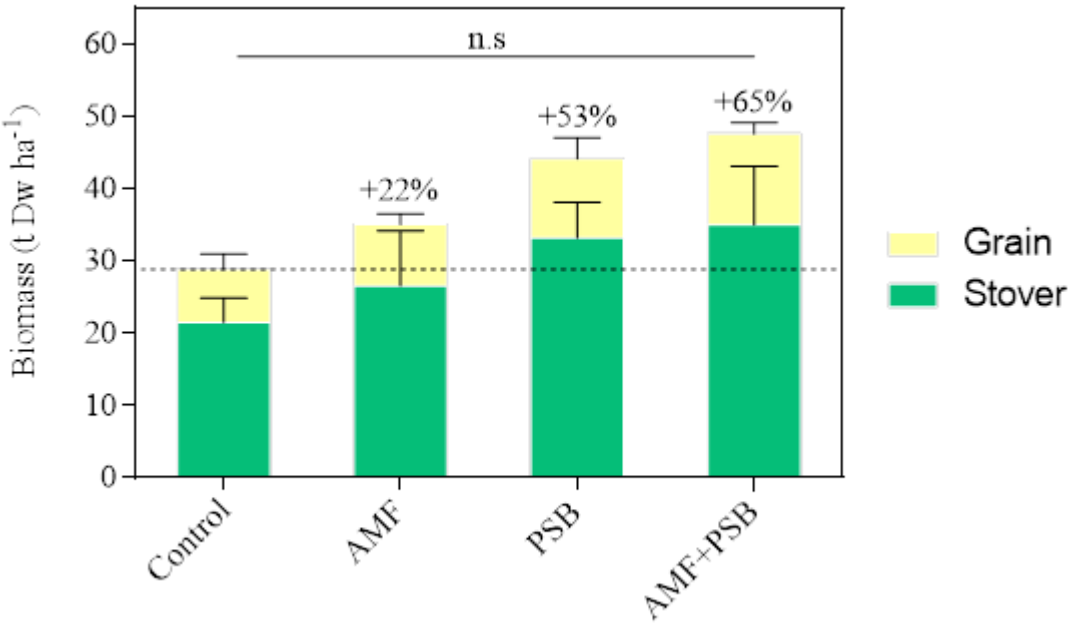

Figure 2.4 Effect of three inoculants (AMF, PSB and AMF+PSB) on maize biomass (shoot) tested under field conditions. Stacked bars (green and yellow) represent the partition of the average dry weight of the stover (culm and cobs) and grain. Values shown at the top of the bars refer to the average percent of increment on maize biomass promoted by the respective inoculant when compared to the control plants. Each bar represents the mean of 3 sampling plots ± SD (n=3). "n.s" indicates there is no significant difference between the treatments at 5% level (for total biomass), according to Tuckey's HSD test. The dashed line marks the mean value of the dry weight of maize biomass of the control plants.

To check if our results resembled a real agricultural scenario, we compared the green forage per plant obtained in this experiment with the average plant green forage for the same soils and climate conditions. Control plants weight more than expected, therefore, we normalized our data for the Portuguese standard average Fw of a maize plant, which is 1.0 kg (Syngenta; Cavaco and Calouro 2006) (Figure 2.5). In this way, the ratio among treatments and control is maintained, but we were able to observe how the weight of each plant changes if the control plants weighted 1.0 kg. In this case, PSB and AMF+PSB inoculants could stimulate plants to reach 1.4 kg and 1.5 kg on average, respectively, when compared to the 1.0 kg control plants (ANOVA Maize GF $F_{3,6}$=9.90, P≤0.01).

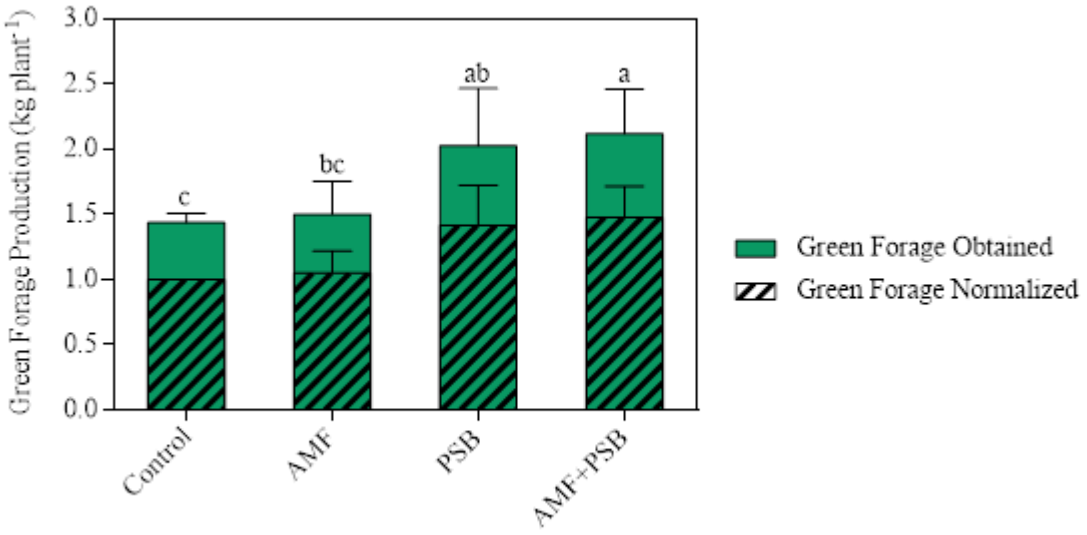

Figure 2.5 Effect of three inoculants (AMF, PSB and AMF+PSB) on maize green forage average weight per plant. Bars in green represent the average green forage obtained per plant in kg and striped bars represent the normalized values of green forage. Data was normalized against the Portuguese standard average fresh weight of a maize plant, which is 1.0 kg. Different letters (mean ± SD of 3 sampling plots, n=3) show significance at 5% level, according to Tuckey's HSD test.

When examining the plants treated with the inoculants, we noticed a trend towards lower dry matter content of the green forage, associated with an increment of Fw (Table 2.1 and Figure 2.3). Therefore, we analysed the effect of the inoculants on the accumulation of dry matter content, but no significant difference was observed (ANOVA dry matter content of the green forage $F_{3,6}$=1.95, P>0.05), even though those were the treatments with higher productivity.

The plants inoculated with AMF+PSB had, on average, more tillers (ANOVA average tillers per plant $F_{3,6}$=18.07, P≤0.01), and consequently, more ears per plant when compared to the control plants (ANOVA average ear per plant $F_{3,6}$=5.34, P≤0.05) (Appendix 3). Plants inoculated with PSB showed the same trend, but without significant differences in relation to other treatments regarding ear production.

Table 2.1 Effect of three inoculants (AMF, PSB and AMF+PSB) on maize dry matter content of the green forage, the average tillers per plant and the average ears per plant. For each column, different letters (mean ± SD of 3 sampling plots, n=3) show significance at 5% level (no letters means not significant), according to Tuckey's HSD test.

Treatment	Dry Matter Content (%)	Average tillers per plant	Average ears per plant
Control	36.8 ± 5.6	0.3 ± 0.6 **b**	1.0 ± 0.0 **b**
AMF	30.9 ± 2.0	0.8 ± 0.4 **b**	1.3 ± 0.6 **ab**
PSB	30.3 ± 5.2	1.7 ± 0.6 **a**	1.9 ± 0.8 **ab**
AMF+PSB	30.7 ± 3.0	1.9 ± 0.2 **a**	2.1 ± 0.5 **a**

2.3.2. Effect of inoculants on P extraction and P fertilizer recovery efficiency

PSB-inoculated plants had 60 % more P in the shoot (stover and grain) when compared to the control plants (ANOVA shoot P extraction $F_{3,6}$=4.88, P≤0.05). However, no significant difference was detected among the other treatments (Table 2.2). Part of this extra P was allocated into the stover. Stover from plants treated with PSB had approximately 31 % and 49 % more P in the shoot than the control and AMF+PSB treated plants, respectively (ANOVA stover P extraction $F_{3,6}$=8.56, P≤0.05). Although, no differences among treatments were found for grain P extraction (ANOVA grain P extraction $F_{3,6}$=0.36, P>0.05). Thus, the differences in shoot P extraction observed among treatments reflected differences in P content of the stover.

Regarding P fertilizer recovery efficiency, it was higher in plants treated with PSB when compared to the control and AMF+PSB (ANOVA P fertilizer recovery efficiency $F_{3,6}$=4.88, P≤0.05). These results were analogous to shoot P extraction.

Table 2.2 Effect of three inoculants (AMF, PSB, AMF+PSB) on the P extraction at shoot, stover (culm and cobs), and grain level and P fertilizer recovery efficiency. For each column, different letters (mean ± SD of 3 sampling plots, n=3) show significance at 5% level (no letters means not significant), according to Tuckey's HSD test.

Treatment	Shoot P extraction (kg ha^{-1})	Stover P extraction (kg ha^{-1})	Grain P extraction (kg ha^{-1})	P fertilizer recovery efficiency (kg P content shoot kg^{-1} P_2O_5 supplied)
Control	85.5 ± 8.8 **b**	77.8 ±10.9 **b**	25.6 ± 5.7	0.44 ± 0.07 **b**
AMF	115.4 ± 11.3 **ab**	84.2 ± 10.6 **ab**	18.3 ± 0.8	0.60 ± 0.11 **ab**
PSB	136.8 ± 5.6 **a**	102.2 ± 6.4 **a**	22.0 ± 2.9	0.71 ± 0.11 **a**
AMF+PSB	98.4 ± 7.8 **ab**	68.4 ± 7.4 **b**	21.4 ± 0.9	0.51 ± 0.04 **ab**

2.3.3. Inoculants and P utilization

Plants treated with AMF+PSB produced significantly more shoot biomass as well as grain Dw per unit of P present in the shoot, when compared to the plants treated with other inoculants (ANOVA PUE_{Shoot} $F_{3,6}$=12.82, P≤0.01; PUE_{Grain} $F_{3,6}$=13.91, P≤0.01) (Figure 2.6). Therefore, AMF+PSB enhanced plants utilization efficiency.

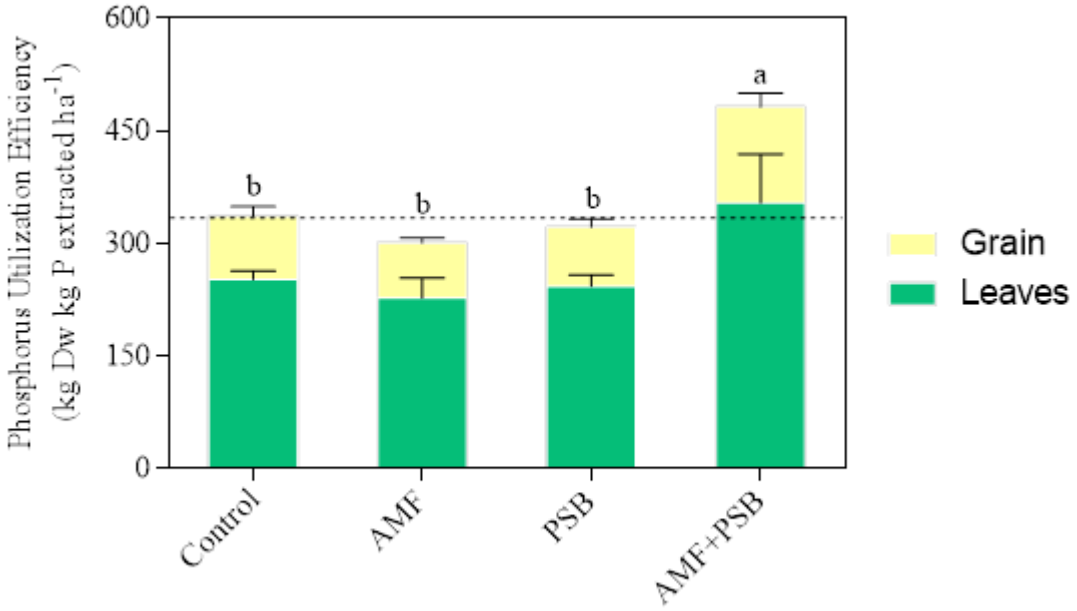

Figure 2.6 Effect of three inoculants (AMF, PSB, AMF+PSB) on P utilization efficiency. Stacked bars (green and yellow) represent the partition of the average PUE at stover (culm and cobs) and grain level. Each bar represents the mean of 3 sampling plots ± SD (n=3). Different letters show significance at 5% level (for total PUE), according to Tuckey's HSD test. The dashed line marks the mean value of the P utilization efficiency of the control plants.

2.3.4. P concentration in plant tissues

Plants treated with AMF+PSB had significantly lower shoot P concentration than plants treated with other inoculants (ANOVA P concentration shoot $F_{3,6}$=17.13, P≤0.01). No difference in shoot P concentration was detected between control, AMF and PSB treated plants (Table 2.3).

P concentration in the stover did not differ between control and inoculated plants (ANOVA P concentration stover $F_{3,6}$=9.60, P≤0.01). However, AMF+PSB plants had lower stover P concentration than AMF and PSB treated plants. Lastly, control plants had the highest P concentration in the grain (ANOVA P Concentration Grain $F_{3,6}$=64.58, P≤0.001). Among inoculated plants, AMF+PSB had lower grain P concentration than AMF inoculated plants. PSB effect did not differ from AMF and AMF+PSB.

In short, inoculants were very efficient in reducing grain P concentration. Although, this difference was not detected in the stover, AMF+PSB treated plants had lower stover P concentration than plants treated with the other inoculants. Overall, AMF+PSB plants were the most efficient in reducing shoot P concentration, a decrease of about 30 % compared to control plants.

Table 2.3 Effect of three inoculants (AMF, PSB, AMF+PSB) on P concentration of shoot, stover (culm and cobs) and grain. For each column, different letters (mean ± SD of 3 sampling plots, n=3) show significance at 5% level, according to Tuckey's HSD test.

Treatment	P concentration Shoot (%)	P concentration Stover (%)	P concentration Grain (%)
Control	0.30 ± 0.01 **a**	0.29 ± 0.02 **ab**	0.32 ± 0.02 **a**
AMF	0.33 ± 0.03 **a**	0.37 ± 0.04 **a**	0.23 ± 0.01 **b**
PSB	0.31 ± 0.02 **a**	0.34 ± 0.03 **a**	0.21 ± 0.03 **bc**
AMF+PSB	0.21 ± 0.03 **b**	0.22 ± 0.04 **b**	0.19 ± 0.01 **c**

2.4. Discussion

Our study allowed the evaluation, under field conditions, of the effects of three inoculants (AMF, PSB and AMF+PSB) on maize growth and P nutrition, and showed that:

i) PSB and AMF+PSB enhanced plant productivity, specifically green forage;
ii) PSB increased P acquisition efficiency, by increasing P content;
iii) AMF+PSB increased P utilization efficiency;
iv) All inoculants affected P allocation within the plant, resulting in lower P concentration in the grain.

2.4.1. Inoculants enhance plant growth

In Portugal, the expected maize production of *FAO 500 Sincere* variety is of 71 – 84 t Fw ha^{-1} (Syngenta), however there are also reports indicating that for P_2O_5 soil content higher than 200 ppm, the expected maize green forage yield should be around 90 t Fw ha^{-1} (Cavaco and Calouro 2006). Regarding biomass production of *Sincere* variety the expected productivity is of 29 – 33 t Dw ha^{-1} (Syngenta). These values are in line with the results achieved in our field trial, 98 t Fw ha^{-1} (Figure 2.3), even though, based on our field expertise, these may be considered high. This high productivity might be associated with favourable environmental conditions, high soil fertility levels, and especially favorable growth conditions early in the season (Cavaco and Calouro 2006).

As expected, inoculants enhanced maize productivity under field conditions (Owen et al. 2015; Ordoñez et al. 2016). However, not all tested inoculants showed a significant difference in productivity in relation to the control plants.

AMF are very important plant symbionts and plant growth promoters. Therefore, it may be surprising that AMF inoculated plants did not promote enhanced performance (Figure 2.3 and Figure 2.4). However, it is well known that advantages provided by AMF to plant development are mainly observed when plants are grown under biotic or abiotic stress conditions (Smith and Read 2008). Regarding the high productivity of the control plants, one can infer that plants were

not under stress, which may justify why plants inoculated with AMF alone did not show the best performance indexes.

The results clearly show that PSB inoculation, with or without AMF, was necessary to obtain an increase in green forage (crop Fw) of 48 % and 41 % respectively, when compared to the control plants (Figure 2.3). Although, when analysing the biomass, no significant difference between inoculants and control plants was verified. Inoculated plants, especially those inoculated with PSB and AMF+PSB, tend to produce bigger plants relatively to the control (Figure 2.4).

We can argue that the distinct effects promoted by the inoculants on green forage and biomass were due to different interactions with the plant water uptake and plant water saving strategies (Richardson et al. 2011). However, it may also reflect the influence of the inoculants in hindering plant development. In fact, in the particular case of cereal plants, both water content and plant development are correlated, since water deficit is one of the main factors involved in starting and accelerating grain production (Varennes 2003; Viderira da Costa et al. 2003). Control plants were harvested with an average dry matter content of 37 % while inoculated plants presented an average dry matter content of 31 %, although differences were not significant.

Plants from this maize variety can grow up to a maximum of 1 kg of green matter and are ripe for being harvested when they achieve a dry matter content of 35 % (Syngenta; Cavaco and Calouro 2006). The control plants, of the present field trial weighted on average 400 g more when harvested with a dry matter content of about 37 %. Thus, in order to estimate productivity increments associated with the inoculants, we normalized productivity to the expected average values (1 kg of green matter per plant). This increment in plant biomass may be related with changes in plant hormonal balance as a result of its interaction with the inoculants (Nadeem et al. 2014). Which is in line with the most intensive plant tillering in plants inoculated with PSB (Banik and Dey 1981; Afzal, A. F. T. A. B., Ashraf, M., Asad, S. A., & Farooq 2005) and AMF+PSB (Chinnusamy et al. 2006). Exogenous hormones regulate the growth of tiller buds by affecting endogenous hormonal levels, thus regulating the occurrence of tillers (Cai et al. 2018).

2.4.2. Inoculants and phosphorus efficiency

On one hand, PSB treated plants were the most efficient at exporting P to the shoot and this difference was verified at the stover level (Table 2.2). P in the stover is present mostly as Pi which is essential in the diets of livestock (Richardson et al. 2011). Normally, dietary P supplements are provided, sometimes in excess, owing to the low availability of P in forages or livestock low P assimilation efficiencies (due to surplus of phytate) (Sharpley et al. 2000). Therefore, PSB could promote not only provender of higher P nutritional value, but also contribute to a reduction on P feed supplements and, consequently, lead to a decrease in P losses in livestock excreta. Yet, this was achieved without changing the P content on the grain (Table 2.2 and Table 2.3). This result reflects the dream of any farmer and can be used as an excellent argument for working more with this isolate towards the development of a biofertilizer based on it.

On the other hand, AMF+PSB treated plants were the most efficient on producing more shoot and grain Dw per unit of P in the shoot, therefore higher PUE (Figure 2.6). This is of extreme importance, not only to help strengthen food production per unit of area, but also to manage the amount of P applied in the farming systems. From the perspective of farm sustainability this would be the most relevant of the tested inoculants.

Higher PUE, can be achieved by plants with lower P concentrations (Rose et al. 2011; Veneklaas et al. 2012). In our experiment, all the treated plants had shoot P concentration (Table 2.3) similar to those recommended for regular plant growth and development, which can range between 2 – 5 g P kg^{-1} of plant Dw (Varennes 2003). The amount of phytate present in plant seeds and grains ranges from 0.5 – 5 g per 100 g of Dw and ideally it should be reduced to 0.025 mg or less per 100g of Dw in order to minimize P losses (Coulibaly et al. 2011). Nevertheless, the AMF+PSB inoculated plants had 30 % less P concentration in the shoots when compared to the control, reinforcing the hypothesis that these plants used less P to produce the same biomass as the other treatments. When analysing the partition of P in the plants, we noticed that the inoculants changed P distribution within the plant. PSB inoculated plants had higher stover P concentration than AMF+PSB, but no difference in P grain concentration was verified (Table 2.2 and Table 2.3). In addition, all inoculants significantly reduced grain P concentration. Most of the P present in the grains is in the form of phytate, which cannot be assimilated by single compartment stomach animals and, therefore, is lost to the environment. Phytate decreases mineral absorption in animals with one stomach (e.g. humans) because phytic acid has a strong ability to bind with ions, such as zinc, calcium, iron and magnesium (Coulibaly et al. 2011). The binding can result in very insoluble salts with poor bioavailability and, therefore, lowering P grain concentration can benefit grain quality by increasing its nutritional value and, consequently, reduce the environmental impacts associated with the loss of P through excreta (Veneklaas et al. 2012).

In short, from the two biofertilizers that increased crop productivity, AMF+PSB are the most efficient in promoting plant growth per unit of P in the tissue. This may be an important trait in increasing grain nutritional value. This inoculant could, in the future, be tested as a way to reduce P inputs, ideally by 30 %, as recommended by the EU, to see if productivity is maintained. Therefore, this AMF+PSB consortium could be a key contributor in producing high value food resources with zero increase in land degradation while reducing negative environmental impacts. Lastly, we must emphasize that even though PSB are not the most efficient in promoting plant biomass production, it is in fact the most efficient in improving P stover content and, therefore, the nutritional value of maize green forage by increasing the content of P in the stover and reducing the concentration of P in the grain, which could be extremely useful in farms where the P availability is really low.

Chapter 3 – The Relevance of Bacteria Consortia in Phosphorus Efficiency

3.1. Introduction

Soil microorganisms are important ecosystem components, and can regulate its productivity and maintain biodiversity (Schnitzer and Klironomos 2011).

Plant-microorganism interactions are an integral part of properly functioning ecosystem, namely regulatory mechanisms that maintain its stability. When these biological processes are inadequate, a drastic decrease in the microbial community can occur, consequently destabilizing the entire ecosystem (Morgan et al. 2005). This is of particular concern in highly managed agricultural systems, where the soil is the first to be destroyed by high levels of fertilizers, pesticides and herbicides, soil mobilisation and lack of plant rotation (Tilman et al. 2002; Stoate et al. 2009). These factors contribute to the reduction of the natural soil microbial community which can threaten agricultural ecosystem productivity and the sustainability of nutrient cycle (e.g. P biogeochemical cycle) (Matson et al. 1997; Tilman et al. 2002). Furthermore, this can change or diminish the services that the agricultural ecosystem can provide. Some studies suggest the inoculation of microorganisms in agricultural fields, such as arbuscular mycorrhizal fungi (AMF) and bacteria, can help to overcome these negative effects on the ecosystem that consequently affect the agricultural productivity (Mahdi et al. 2010; Bhardwaj et al. 2014; Nadeem et al. 2014).

Inoculation of multistrain biofertilizers have been shown to increase yields under field conditions (Cong et al. 2011). Other studies showed that it was possible to reduce synthetic fertilization inputs using multistrain biofertilizers (Rose et al. 2014).

The symbiotic association between plant roots and AMF has long been established as an important mechanism by which plants are able to acquire P (and other nutrients) from the soil (Gerdemann 1970; Smith and Read 2008). AMF can transport P to the plant which in some cases adds up to 70 % of total P plant uptake (Richardson 2001). However, a big part of P present in the soil is in an insoluble form and AMF can only exploit soluble P sources (Ordoñez et al. 2016). Therefore, its association with other groups of microorganisms could improve its performance and stimulate a more varied range of functions.

Mycorrhiza and bacteria can act as cross facilitators, meaning that they can increase the fitness of each other (Manchanda et al. 2017). To provide suitable ecological niches and nutrition for bacteria, AMF may influence cohabiting species via the secretion of chemical substances. The nature of these substances may define the type of interaction between AMF and bacteria. Bacteria improve the mycorrhization, provide a pool of available P and N, and help in management of biotic (e.g. pathogen) and abiotic (e.g. nutrient unavailability) stresses (Smith et al. 2011; Manchanda et al. 2017). In particular, P solubilizing bacteria (PSB) such as *Pseudomonas spp*., are known to colonize the rhizosphere, and can exhibit additional plant growth promoting characteristics such as plant growth stimulation and the production of metabolites that have anti-microbial activity (Khan et al. 2009a). Most results from experiments conducted in sterilized soil demonstrate that AMF and PSB act synergistically. Recent evidence also points not only to synergistic effects between AMF and PSB but also to cooperation between these organisms.

Through cooperation, AMF and PSB can get what they need from their partners and improve their own fitness, like a reciprocal reward mechanism (Zhang et al. 2016). However, most of the beneficial effects were observed in experiments conducted in sterile soil, *in vitro* or microcosm.

The predictability of the efficacy of biofertilization is one of the most challenging subjects nowadays in achieving a more sustainable agriculture (Owen et al. 2015). Throughout the past years, a large range of results has been published concerning microbial biofertilization (Vessey 2003; Fuentes-Ramirez and Caballero-Mellado 2006; Bhardwaj et al. 2014). However, the application of biofertilizers in agriculture has had very variable success (Herrmann and Lesueur 2013; Owen et al. 2015), being inconsistent and contradictory, which may discourage companies to allow their products to undergo a rigorous scientific examination. The main concerns that arise are the compatibility between microorganisms in the biofertilizer, as well as their interactions with plants and the microbial community present in the soil (Owen et al. 2015). Moreover, the reproducibility of biofertilizers' effects is still a challenge due to the lack of consistency in results obtained under field conditions (Herrmann and Lesueur 2013; Schmidt and Gaudin 2018).

Studies suggest that through microbial inoculation, such as multistrain or diverse microbial consortium, the functions of soil microbiota can be re-established and, consequently, the productivity increased (Wu et al. 2005; Owen et al. 2015; Ordoñez et al. 2016). This goes along with the "niche complementary hypothesis" which postulates that increasing species diversity can enhance productivity because it leads to a more efficient acquisition of limiting resources (e.g. nutrients) by each added species and consequently promotes a use of whole-ecosystem resources (Fargione et al. 2007; Schnitzer and Klironomos 2011). However, at high diversity, the resource requirements of additional species overlap with existing ones and, consequently, productivity no longer increases with diversity, resulting in the asymptotic diversity-productivity pattern (Schnitzer and Klironomos 2011). Contrariwise, other authors state that ecosystem properties are mainly defined by the species composition, rather than species diversity (Tilman 1997).

Having in mind the problematic of global P resources depletion and high P demanding agricultural practices, novel strategies regarding P use efficiency are needed, as has been encouraged by the European Union (EU).

AMF+PSB show a potential to increase plant growth and P utilization efficiency as demonstrated in Chapter 2. As such, with this pot experiment, it is proposed, as the main goal, to test whether increasing species richness and composition in bacterial consortia (adding different species of PSB in combination with AMF) would potentiate plant growth and P use efficiency when P fertilization is reduced by 33 %, according to EU recommendations.

It was expected that a more diverse bacterial consortium would be more efficient at promoting plant growth and P use efficiency, attaining similar plant response as though subjected to full regular mineral fertilization.

3.2. Material and methods

3.2.1. Growth conditions

The study had a duration of 70 days, was conducted in the greenhouse park of the Faculdade de Ciências, Universidade de Lisboa, Portugal (38° 45' 29.2" N 9° 09' 29.5" W), from April to June of 2017. Daily mean air temperature was 23 ºC and ranged between 11 – 42 ºC and relative

humidity between 18 – 93 % (Data was obtained with EasyLog USB daily every hour) (Appendix 4).

3.2.2. Soil collection and analysis

The soil used in this experiment was collected from an agricultural field, located in Arrábida, Setúbal district, Portugal. The soil was air-dried and sieved through an 8 mm mesh sieve, then mixed in a 1:1 (v/v) ratio with washed sand (particles with less than 4 mm), to create a nutrient poor soil. The basic properties of the resulting soil mixture were as follows: organic matter content 0.38 %, pH (H_2O) 6.8 and P content of 44 ppm (Method of Egnér-Riehm) (Laboratório de Solos e Plantas, UTAD 2017). Pots were filled with 2 kg (dry weight) of the soil mixture.

3.2.3. Plant species: seed germination

Zea mays L., *FAO 500* variety from *Syngenta* was used as crop material. Plants were grown for approximately 3 weeks in sterilized vermiculite substrate on a seed tray. Twice a week, seedlings were supplied with sufficient water to maintain an average substrate moisture (equivalent to 40 – 60 % water pore space capacity). Seedlings with an average fresh weight of 3 g and shoot length of 20 cm, were transplanted into pots. Seed remains were removed before transplantation to maximize the treatment effect (Figure 3.1). Each pot was planted with a single seedling. After transplantation a 7-day period was checked for transplantation shock, allowing seedling substitution if necessary. The experimental time started counting after this period. Plants were watered three times per week to field capacity. All pots were randomly placed in the greenhouse and randomly changed place every week.

Figure 3.1 Seedling prepared for pot transplantation where seed was removed (indicated with an arrow).

3.2.4. Experimental design

The microbial consortium providing the best results in the field experiment (AMF+PSB1; Chapter 2) was supplied with one or two other PSB (*Soilvitae* collection) in order to assess the effect of increased PSB (1, 2 or 3) species diversity on inoculant efficiency and plant performance under 67 % P fertilization dose. The following controls were performed: AMF+PSB2,

AMF+PSB3 (67 % P fertilization dose); and no inoculation using 67 and 100 % P fertilization dose.

All the PSB inoculants were distinct *Pseudomonas sp.* species, with similar P solubilization potential, isolated from Portuguese agricultural soils. These bacteria were characterized as PSB due to their capacity to solubilize tri-calcium phosphate and phytate in *in vitro* conditions.

The experiment had a completely randomized design, where treatments were the result of six combinations of microbial inoculants under 67 % P fertilization. The combinations between AMF and PSB included the addition of one (i), two (ii) and three (iii) species of PSB. Control groups lacking inoculants were grown under 67 % and 100 % P fertilization (iv). Five replicates per treatment were performed, totalling 40 pots. The treatments were as follows:

(i) AMF+PSB1, AMF+PSB2, AMF+PSB3;
(ii) AMF+PSB(1+2), AMF+PSB(1+3);
(iii) AMF+PSB(1+2+3);
(iv) Uninoculated control 100 % P fertilization (UC 100) and uninoculated control 67 % P fertilization (UC 67).

3.2.5. Microbial consortia inoculation

AMF spores were isolated and counted from a commercial product to estimate the amount of product added to each pot (Appendix 2). At transplantation seedlings were inoculated with approximately 150 - 200 spores/plant of *R. irregulare* by delivering the spores into the plantation hole (10 cm depth). Seven days later the PSB inocula (1 mL of 10^9 CFU mL^{-1} of all bacteria pertaining to each treatment) were added to plant roots. This first PSB inoculation was considered the beginning of the experiment (T_0). A second (T_1) and third (T_3) plant inoculations with the same PSB dose used at T_0 were performed one and three weeks after, respectively.

3.2.6. Mineral Fertilization

Soil fertilizer was previously mixed with the soil. Rock phosphate (RP), $Ca_3(P_2O_5)$, which contained 32 % P in the form of P_2O_5, was used as P source. Two P levels were tested (67 % and 100 %). P 67 % corresponded to a dose of 314 mg of RP kg^{-1} soil (100 ppm P_2O_5) and P 100 % corresponded to a dose of 470 mg of RP kg^{-1} soil (150 ppm P_2O_5). The remaining nutrients (Nitrogen, Potassium and micronutrients) were equally applied in all treatments. All plants were fertilised with 38 mg NO_3^-, 38 mg NHO_4^+, 10 mg CaO and 3 mg MgO kg^{-1} soil at T_0 and two weeks after (T_2) the start of the experiment (ADP Fertilizantes 2016). 100 mL of a ¼ strengh Hogland's solution (Dias et al. 2018) without P (1.5 mM KNO_3; 1 mM $Ca(NO_3)_2$; 0.25 mM $MgSO_4$; 50 µM KCl; 25 µM H_3BO_3; 2 µM $MnSO_4$; 2 µM $ZnSO_4$; 0.5 µM $CuSO_4$; 0.5 µM $(NH_4)_6Mo_7O_{24}$; 20 µM FeNaEDTA) was applied, to fill the nutritional needs of the plant, at T_3 and nine weeks after (T_9) the start of the experiment, which represented the addition of 5.6 mg N, 6.0 mg K, 4.0 mg Ca, 0.6 mg Mg, 0.8 mg S, 27.5 µg B, 177.5 µg Cl, 3.2 µg Cu, 112 µg Fe, 11 µg Mn, 33.6 µg Mo, and 13.1 µg Zn per application. Zinc deficiency was detected during the experiment, as such additional Zinc was added spaced six times over the experiment, which represented the addition of 13.1 µg Zn per application.

3.2.7. Data collection and analysis at harvest

Plant height is an important component of fitness because it improves access to light, which can subsequently maximize reproductive success (Falster and Westoby 2003). Plant height, distance from the soil surface to the uppermost extended leaf tip was measured, at seedlings transplantation into pots, then at the beginning of the experiment T_0, five (T_5), eight (T_8) and ten (T_{10}) weeks after the the experiment started.

Leaf area at harvest sampling was estimated through the following process: leaves from each plant were separated from the shoot and arranged on a flat surface with a ruler next to it. A camera support was placed 1m above the blue cardboard and images were recorded with a phone camera (Huawei Ascend G6). Every image was taken at the same distance, to minimized scale errors. ImageJ (FIJI) was used to calculate leaf area, as shows in Appendix 5. This program uses a threshold-based pixel count measurement to calculate areas.

Shoots and roots were cleaned and fresh weight (Fw) determined. Dry weight (Dw) was measured using a precision weighting scale (precision 0.01 / 0.001, model CM-300 CBJ), after drying plant material at 65ºC for 3 days. Total plant biomass was calculated by adding shoot and root Dw.

From the five replicates per treatment, a sub-sample of 3 plants, randomly chosen, was reduced to powder using a mill of spheres. The resulted samples were used to perform root and shoot analyses to determine P concentration, using an Optical Emission Spectroscopy after acid digestion (Huang and Schulte 1985) (analysis performed by Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), Murcia, Spain 2016).

Phosphorus acquisition efficiency (PAE) which represents the amount of P uptaken per plant (Wang et al. 2010; Vandamme et al. 2016) was calculated through shoot P content and P fertilizer recovery efficiency, as follows:

(3.1)

$$P\ content = Dry\ weight\ tissue \times P\ tissue\ concentration$$

(3.2)

$$P\ fertilizer\ recovery\ efficiency = \frac{P\ content\ plant}{P\ applied\ in\ the\ pot}$$

P content (g plant^{-1}) reflects the total P present in plant tissues: shoot and root, which was extracted from soil. This was calculated by combining root (g) and shoot (g) Dw with respective P concentration (g P/100 g plant) for each treatment, as depicted in formula (3.1). Plant P content was obtained through the sum of root and shoot P content, while plant concentration was obtained by dividing plant P content by plant Dw.

P fertilizer recovery efficiency (g plant^{-1}) reflects the ability of a plant to acquire nutrients applied to the soil (Baligar et al. 2001) and was calculated by shoot P content (g) divided by the amount of P fertilizer applied (g pot^{-1}), as depicted in formula (3.2).

Phosphorus Utilization Efficiency (PUE) was evaluated through the amount of Dw (shoot or root Dw) produced per unit of P present in the plant (plant P content) (Rose and Wissuwa 2012), as follows:

(3.3)

$$PUE = \frac{Dry\ weight\ tissue}{P\ content(plant)}$$

PUE (g Dw g P content plant^{-1}) was expressed in g of tissue biomass produced per g of P present in the plant, formula (3.3).

The physiological responses of the plants to the different treatments were assessed using five standard measures of leaf/plant performance. These measures of plants performance were relative leaf chlorophyll (LC) content, Normalised Difference Vegetation Index (NDVI), Photochemical Reflectance Index (PRI), Carter Index 2 (CTR2) and Water Index (WI).

A relatively efficient and scorable physiological parameter is LC, which is affected by plant and P stress and can be measured *in vivo* using a portable device, SPAD meter (SPAD-502 Plus) (van de Wiel et al. 2016). The SPAD-502 Plus measures the transmittance of light through the leaf at 650 nm, which corresponds to the maximum region of absorption of chlorophyll. A second light source at 940 nm allows internal calibration. In this way, the apparatus provides dimensionless estimates of the chlorophyll content. SPAD-502 Plus uses its own scale of -9.9 to 199.9 SPAD values, a low value indicates low LC content and a high value indicates higher LC content. Five measurements were performed per plant on the youngest full developed leaves. The measurements were performed five (T_5) and nine (T_9) weeks after the start of the experiment.

NDVI, PRI and CTR2 measures were taken with a PolyPen RP 400® (Photon Systems Instruments, Drazov, Czech Republic) in the youngest fully developed leaf per plant. The PolyPen is used to measure the spectral reflectance, light transmittance and light absorbance of leaves (Photon Systems Instruments, 2014). The device uses spectral reference of an internal light source at 380 – 1050 nm wavelength to measure light reflectance and absorbance. The device then uses incorporated formulas (based on specific light wavelengths, listed below) for commonly used light reflectance indexes. The measurements were performed two (T_2) five (T_5) and nine (T_9) weeks after the start of the experiment.

The instrumental formula for NDVI calculation is:

(3.4)

$$NDVI = \frac{R_{\mathrm{NIR}} - R_{\mathrm{RED}}}{R_{\mathrm{NIR}} + R_{\mathrm{RED}}}$$

The NDVI combines the information available in the red (R_{RED}) and near-infrared (R_{NIR}) bands into a single representative value. Reflectance in the red spectral band is subtracted from reflectance in NIR and divided by the sum of the NIR and red reflectance, as depicted in formula

(3.4) (Rouse, J. W. et al. 1974). Light is absorbed by chlorophyll during photosynthesis, in the red region (~625 – 700 nm) of the electromagnetic spectrum, and near-infrared light (~700 – 1100 nm) is absorbed by the internal cellular structure of the leave or biomass of the plant. NDVI values range between -1 and 1. A higher NDVI value (e.g. between 0.5 to 0.9) indicates healthy leaf function and a lower NDVI value (e.g. between 0.1 and 0.4) indicates unhealthy leaf function (i.e. diminished or lack of leaf function) (Yoon and Thai 2010).

The instrumental formula for PRI calculation is:

(3.5)

$$PRI = \frac{R_{531} - R_{570}}{R_{531} + R_{570}}$$

Where R_{531} indicates reflectance of wavebands of light at 531 nm (the waveband of the "xanthophyll signal") and R_{570} is used as a waveband reference, which is then used to assess/determine leaf health (Gamon et al. 1997). Formula (3.5). PRI measurements of leaves range between -0.1 and 0.1. A positive PRI value indicates healthy leaf function and a negative PRI value indicates unhealthy leaf function (i.e. diminished or lack of leaf function) (Thenot et al. 2002).

The instrumental formula for CTR2 calculation is:

(3.6)

$$CTR2 = \frac{R_{695}}{R_{760}}$$

Where R_{695} indicates reflectance of wavebands of light at 695 nm (far red region of the spectrum) and R_{760} indicates reflectance of wavebands of light at 760 nm (infrared region of the spectrum), as depicted in formula (3.6) (Carter 1994). High CTR2 indicates more stress.

WI measures were taken with UniSpec-SC is a single channel (VIS/NIR), portable instrument commonly referred to as a "leaf reflectometer". It is an ideal instrument for measurement of leaf level reflectance on individual leaves because it allows to obtain measurements in full light spectrum (310 - 1100 nm). The measurements were performed in the youngest fully developed leaf per plant at five (T_5) and nine (T_9) weeks after the start of the experiment.

WI index shows plant and water relation. WI was calculated as follows (Penuelas et al. 1997):

(3.7)

$$WI = \frac{R_{900}}{R_{970}}$$

Where R_{900} indicates reflectance of wavebands of light at 900 nm and R_{970} indicates reflectance of wavebands of light at 970 nm, as depicted in formula (3.7). This ratio is highly correlated with plant relative water concentration and high WI values indicate more stress (Penuelas et al. 1997).

Soil samples were collected at the beginning of the experiment (bare soil). Samples were air-dried and then were analysed for chemical and physical properties: extracted P (Egnér-Riehm method), organic matter quantification and pH (H_2O) (analysis performed by Laboratório de Solos e Plantas, UTAD, Portugal, 2016).

3.2.8. Statistical analysis

The effect of the inoculants on maize performance was tested via one-way analysis of variance (ANOVA). Differences among treatment means were determinate by Tukey test ($p \leq 0.05$). Homoscedasticity was tested using the Levene's test. The statistical analysis was achieved by analysis of variance that included main effects of treatments, using the software IBM SPSS Statistics version 25. Graphs were developed with GraphPad Prism version 6 (GraphPad Software, San Diego, CA).

A principal component analysis (PCA) was carried out using the software R version 3.5.1.

Leaf area data had some extreme outliers, detected using an outlier detection test, which were removed.

3.3. Results

3.3.1. Effect of different consortia on plant growth

To evaluate the effect of inoculants on maize performance, plant dry weight (Dw), plant height and leaf area were assessed.

The total plant Dw (ANOVA $F_{7,32}=9.53$, $P \leq 0.001$) as well as the shoot (ANOVA $F_{7,32}=10.07$, $P \leq 0.001$) and root (ANOVA $F_{7,32}=6.81$, $P \leq 0.001$) Dw were affected by the inoculants. Comparing uninoculated plants, reduced P fertilization (UC 67) resulted in plants with less 28 % Dw than full P fertilization (UC 100) (Figure 3.2a). Showing that UC 67 plants were P limited.

Plant inoculation with AMF and a single species of bacteria (PSB1, PSB2 or PSB3) did not produce differences in the plant Dw compared to control treatments (UC 100 and to UC 67).

At 67 % P fertilizer dose, plants inoculated with AMF+PSB(1+2) had 40 % less Dw than non-inoculated plants (UC 67). Yet, in the other consortium with two PSB (AMF+PSB(1+3)) there were no significant differences in plant Dw relatively to the non-inoculated plants (24 % less than UC 100 and 7 % more than UC 67). The inoculation of AMF with the three bacterial species (PSB1+2+3) showed a 47 % increment in the plant Dw when compared to the UC 67 plants; and a tendency for slightly higher Dw when compared to the full fertilization control treatment (UC 100). Root and shoot Dw (Figure 3.2b), were affected by the treatments in a similar way to that of total plant Dw. Overall, these results show that increasing species richness and

composition of different microbial consortia did not always increase the growth of maize plants (Table 3.1).

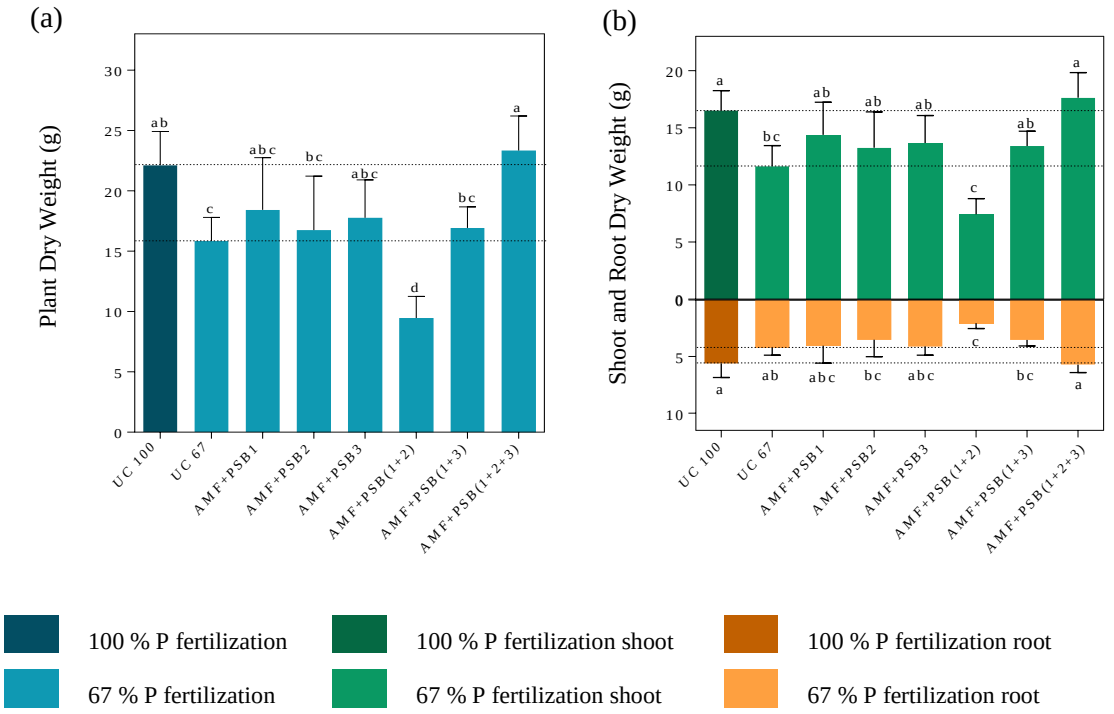

Figure 3.2 Effect of six microbial consortia (one AMF species combined with different species of PSB) on (a) plant dry weight and (b) shoot and root dry weight, tested on pot conditions. Bars show the average dry weight of the plant structures. Green bars represent shoot while brown bars represent root. Darker bars symbolise 100 % P fertilization while lighter bars represent 67 % of P fertilization. Bars represent the mean of 5 replicates ± SD (n=5). Different letters show significance at 5% level, according to Tuckey's HSD test. The dashed line marks the mean value of the dry weight of maize of the uninoculated control plants (UC 100 and UC 67).

Table 3.1 Growth effect, positive (+) in blue and negative (-) in red, of six microbial consortia (one AMF species combined with different species of PSB) on plant biomass.

Treatment	Growth Effect
AMF+PSB(1+2+3)	+
AMF+PSB(1+2)	-
AMF+PSB(1+3)	+
AMF+PSB1	+
AMF+PSB2	+
AMF+PSB3	+

During the first 5 weeks (T_5) of the assay plant height increase was very low, probably due to low temperatures for maize growth (Figure 3.3). Between T_5 and $T_{8,}$ AMF+PSB(1+2), which was the treatment with the lowest height increment (based on plant Dw), did not show an increase in the average plant height. However, between T_8 and T_{10} this treatment was able to recover its growth but, it continued to be the treatment with lower performance when compared to UC 100 (ANOVA plant height T_{10} $F_{7,32}$= 5.64, $p \leq 0.001$). AMF+PSB(1+2+3) was the treatment with most stable growth rates. This treatment seemed not to be affected by the variables that slowed down the growth rate of the other treatments (i.e.: temperatures, P availability, etc…). The highest height increment was between the last 2 weeks of the experiment for all treatments. Plants treated with PSB3 did not statistically differ from those of UC 100 or UC 67 treatments in terms of height. However, PSB3 treated plants tended to be taller than UC 67 treated plants.

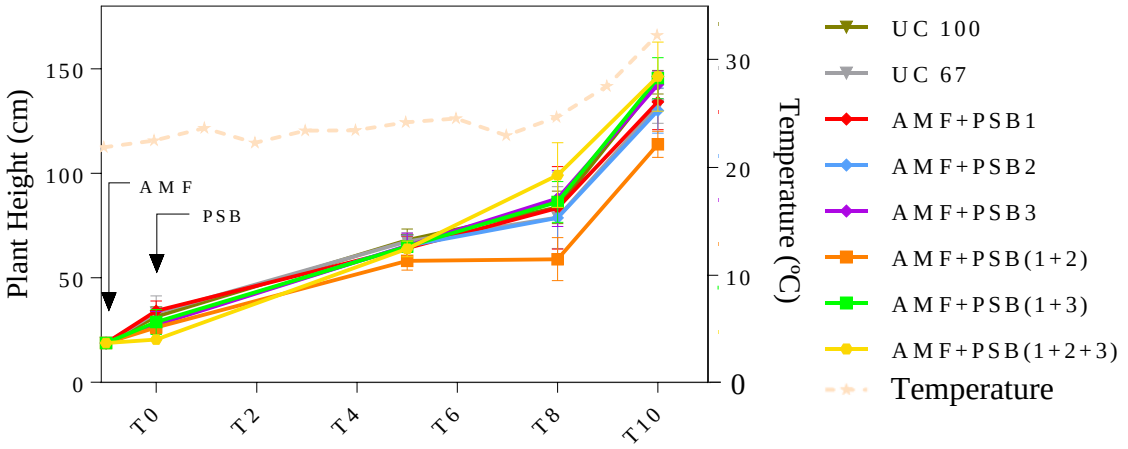

Figure 3.3 Effect of six microbial consortia (one AMF species combined with different species of PSB) on plant height at the five sampling times. Symbols represent the mean of 5 replicates ± SD (n=5). T0 to T10 represent the duration of the experiment in weeks. AMF and PSB additions are indicated with arrows. Temperature depicted represents the mean per week.

In uninoculated plants, reduced P fertilization (UC 67) resulted in a reduction of 27 % of plant leaf area in comparison with full P fertilization (UC 100) (Figure 3.4). A similar pattern to plant Dw (Figure 3.2a). The most diverse consortium, AMF+PSB(1+2+3), promoted leaf area development in 50 % more when compared with that of UC 100 treated plants (One-way ANOVA leaf area $F_{7,26}$= 6.02, p≤0.001). Apart from AMF+PSB(1+2), no significant differences where observed between UC 100 and the other treatments. Once again AMF+PSB(1+2) had the lowest performance presenting a tendency to produce plants with smaller leaf area, 42 % less, compared with UC 67 treated plants.

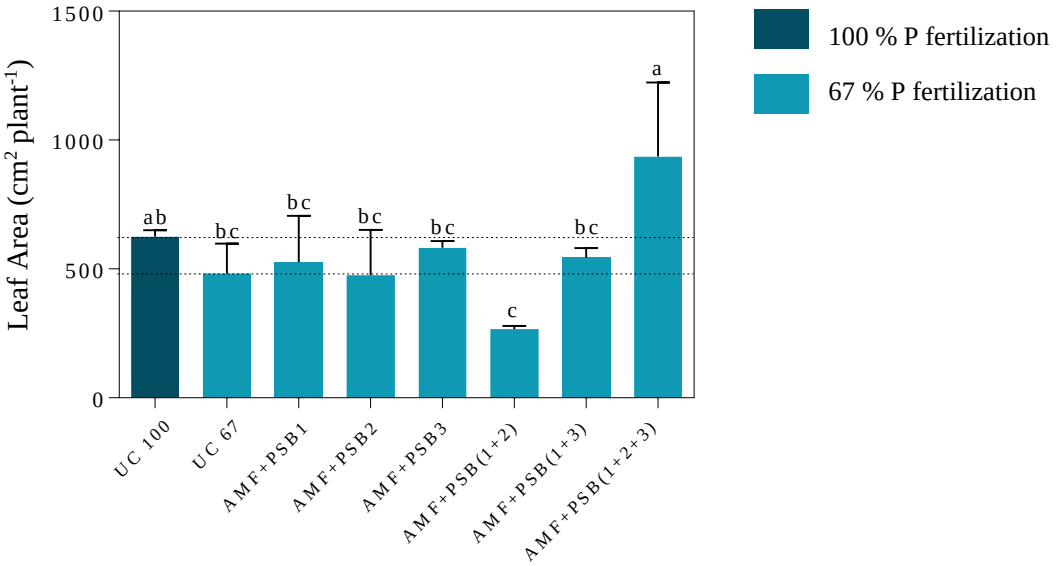

Figure 3.4 Effect of six microbial consortia (one AMF species combined with different species of PSB) on leaf area. Bars show the average leaf area of the plants. Darker bar represents a 100 % P fertilization while lighter bars represent 67 % of P fertilization. Bars represent the mean of 5 replicates ± SD (n=5). Different letters show significance at 5% level, according to Tuckey's HSD test. The dashed line marks the mean value of the dry weight of maize of the uninoculated control plants (UC 100 and UC 67).

3.3.2. P concentration in plant tissues and P efficiency (PAE and PUE)

Shoot P concentration of UC 67 treated plants tended to be higher (in about 26 %) than that of UC 100 treated plants, however this difference was not significant (ANOVA P concentration shoot $F_{7,16}$=2.66, P≤0.05) (Figure 3.5a). Plants treated with a combination of AMF and a single species of bacteria (PSB1, 2 or 3) had similar root and shoot P concentration when compared to UC 67 plants. AMF and two species of bacteria treated plants had a tendency for higher shoot and root P concentration when compared to UC 100 plants, however AMF+PSB(1+2) plants had an increment of shoot in about 90 % in comparison to the uninoculated plants with full P fertilization (UC 100). Regarding root P concentration AMF+PSB(1+2) treated plants showed a tendency for higher values when compared to uninoculated UC 100 and UC 67 (ANOVA P concentration root $F_{7,16}$=3.13, P≤0.05). When comparing plants inoculated with AMF and three species of bacteria with UC 100 plants it was verified that AMF+PSB(1+2+3) treated plants showed a trend for higher shoot P concentration and lower root P concentration.

Regarding shoot P content, as hypothesized, the reduced P fertilization control plants (UC 67) had lower shoot P content in the plant tissues than the full fertilization control plants (UC 100). The difference between the fertilization control treatments represented a decrease of about 11 % in shoot P and 13 % in plant P however no significant difference was observed (Figure 3.5b). Among treatments there was a tendency for the plants treated with AMF+PSB(1+2) to have lower shoot and root P content when compared to the UC 100 plants however no significant difference was observed (ANOVA P content shoot $F_{7,16}$=2.22, P>0.05; ANOVA P content root $F_{7,16}$=5.56, P≤0.01).Treatments containing PSB3 showed a tendency for higher P content in the shoots and seemed to have an overall highest plant P content when compared with the other treatments (ANOVA P content plant $F_{7,16}$=2.52, P>0.05). Perhaps differences in P content are related to the differences in biomass, since AMF+PSB(1+2) are the smallest plants while AMF+PSB(1+2+3) are the largest plants.

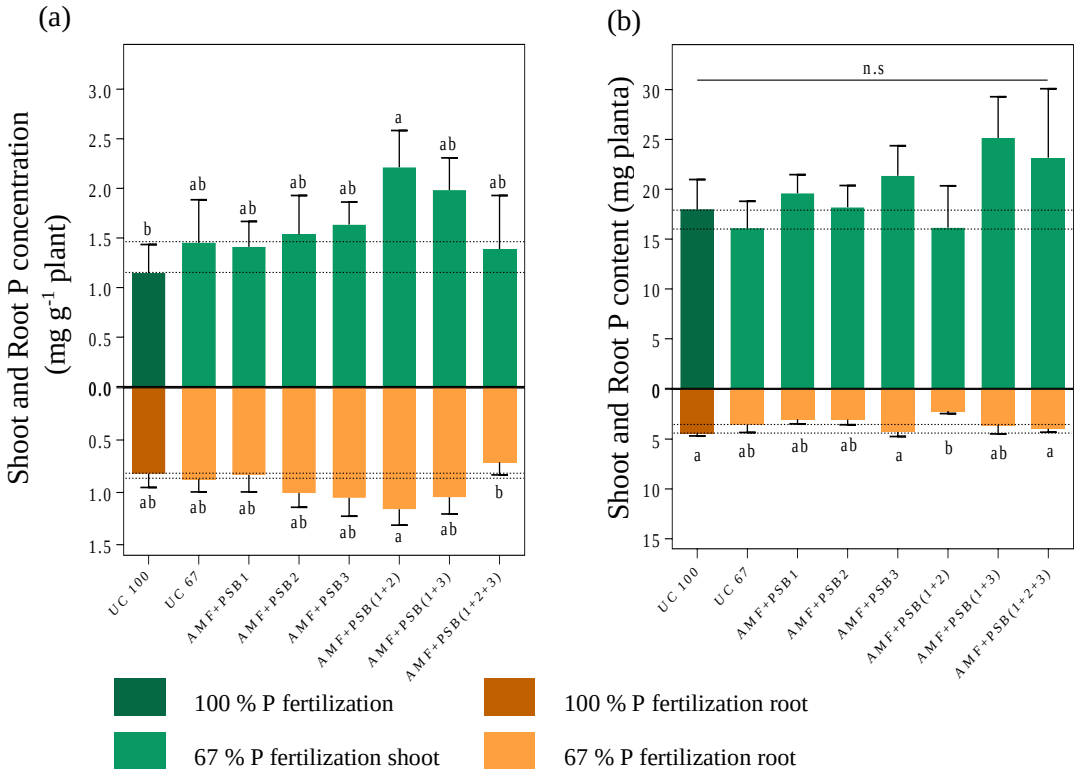

Figure 3.5 Effect of six microbial consortia (one AMF species combined with different species of PSB) on (a) shoot and root phosphorus concentration (b) shoot and root phosphorus content. Green bars represent shoot while brown bars represent root. Darker bars represent a 100 % P fertilization while lighter bars represent 67 % of P fertilization. Bars represent the mean of 3 replicates ± SD (n=3). Different letters show significance at 5% level, according to Tuckey's HSD test. The dashed line marks the mean value of the dry weight of maize of the control plants (UC 100 and UC 67).

Regarding P fertilizer recovery efficiency, plants inoculated with the consortia AMF+PSB(1+2+3) and AMF+PSB(1+3) were the most efficient in acquiring P applied to the soil in the form of RP, when compared to uninoculated control plants with full P fertilization (UC 100), having 82 % and 93 % more P in the plant tissues than UC 100 plants (ANOVA P fertilizer recovery efficiency $F_{3,6}$=4.29, P≤0.01) (Figure 3.6).

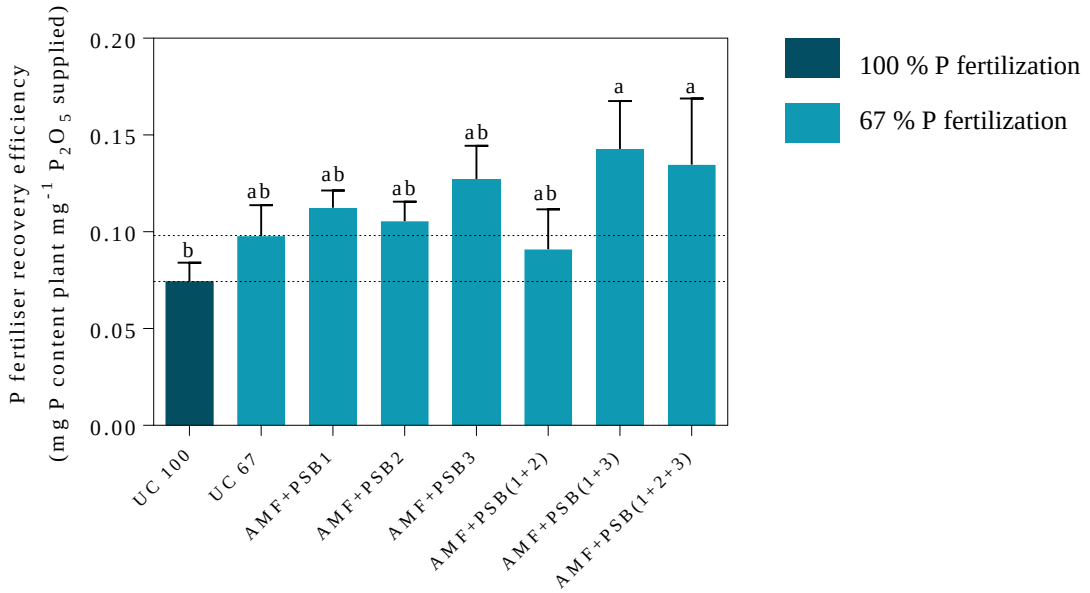

Figure 3.6 Effect of six microbial consortia (one AMF species combined with different species of PSB) on phosphorus fertilizer recovery efficiency. Darker bars represent a 100 % P fertilization while lighter bars represent 67% of P fertilization. Bars represent the mean of 3 replicates ± SD (n=3). Different letters show significance at 5% level, according to Tuckey's HSD test. The dashed line marks the mean value of the dry weight of maize of the control plants (UC 100 and UC 67).

No difference was observed regarding P utilization efficiency among treatments (Figure 3.7). Among the inoculated plants, AMF and 3 species of bacteria, was the treatment that showed a tendency for higher shoot, root and plant PUE (ANOVA PUEshoot $F_{3,6}$=1.82 P>0.05; ANOVA PUEroot $F_{3,6}$=3.09, P≤0.05; ANOVA PUEplant $F_{3,6}$=2.14, P>0.05).

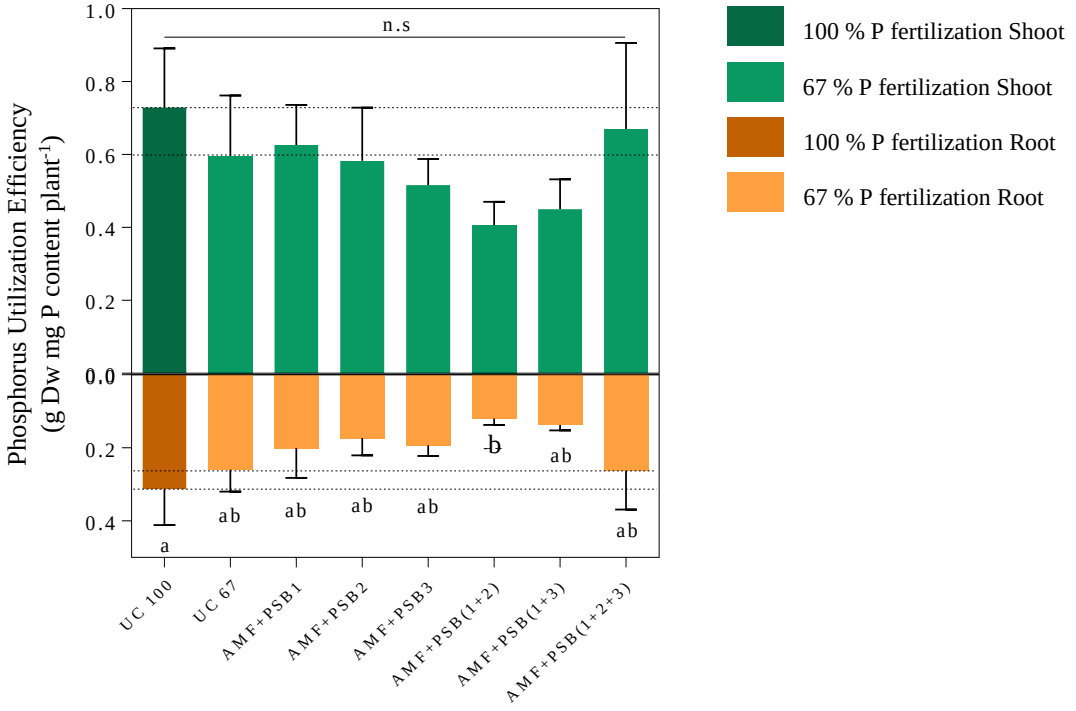

Figure 3.7 Effect of six microbial consortia (one AMF species combined with different species of PSB) on phosphorus utilization efficiency. Green bars represent shoot while brown bars represent root. Darker bars represent a 100 % P fertilization while lighter bars represent 67% of P fertilization. Bars represent the mean of 3 replicates ± SD (n=3). Different letters show significance at 5% level, according to Tuckey's HSD test. The dashed line marks the mean value of the dry weight of maize of the control plants (UC 100 and UC 67).

3.3.3. Physiological response to microbial inoculants

A principal component analysis (PCA) analysis shows plant response (morphologic and physiologic response) to different microbial consortium inoculation. We considered as morphologic response as biomass (Dw plant^{-1}) (T10), plant height (cm) (T8) (Appendix 8) and leaf area (cm^2) (T10) and physiological response the different physiological indexes SPAD, NDVI, PRI, CTR2 and WI (T_5). Since there was a great increase in growth between T5 and T8, we selected the measurements of the physiological indexes at (T5), in order to explain this phenomenon through the physiological indexes.

Factor 1 accounts for 65.8 % of the total variance (Figure 3.8). Plant morphologic (biomass, plant height and leaf area) and plant physiologic (SPAD, PRI and NDVI) responses are directly related, and they all are inversely related to CTR2 (which has a negative value in rotation of PC1). The Factor 2 accounts for 14.6 %, showing as significant variable WI. Treatment are affecting all these variables.

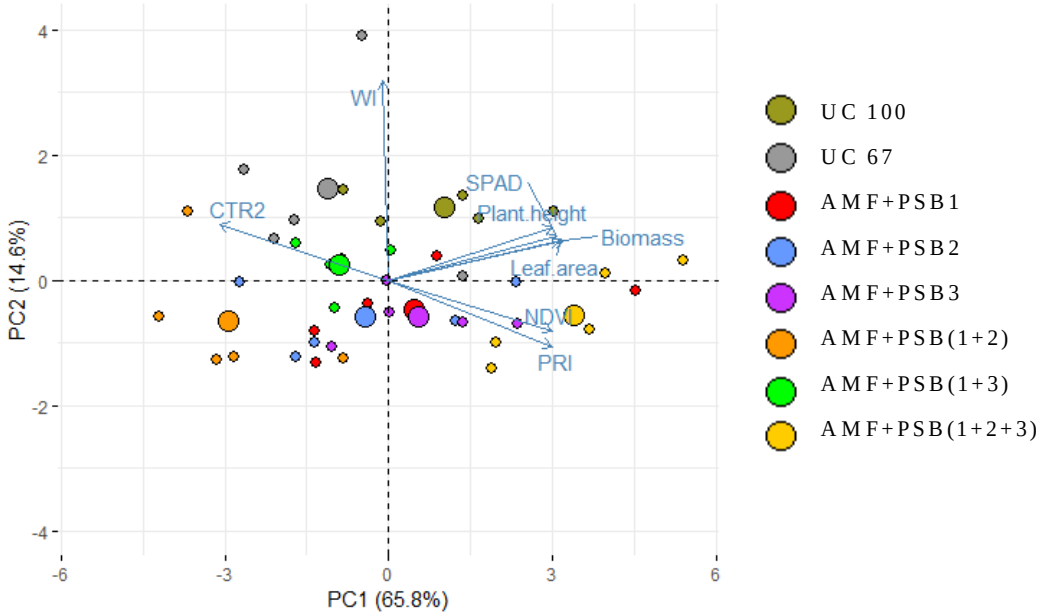

Figure 3.8 Principal component analysis (PCA) biplot (components 1 and 2) of plant response (morphologic and physiologic response) to different microbial consortium inoculation. Vectors represent variables such as plant morphology (biomass, plant height and leaf area) and plant physiology (physiological indexes SPAD, NDVI, PRI, CTR2 and WI). Larger circles represent the centroid for each data group. Variance explained by the first two axes, factor 1 (PC1) accounts for 65.8 % of the total variance and factor 2 (PC2) accounts for 14.6 % of the variance.

At the middle of the experiment (T_5) it is possible to perceive that there were physiological differences among the treatments. Plants that had the best growth performance, had at T_5 the highest SPAD values and NDVI values, which indicates that these plants could have had higher leaf chlorophyll content and high healthy leaf function (ANOVA SPAD T_5 $F_{7,32}$=4.55, P≤0.001; NDVI T_5 $F_{7,32}$=3.50, P≤0.01) (Table 3.2 and Table 3.3). Inoculated plants had lower water index values which can indicates that these treatments allowed plants to be more resilient to possible water stress (ANOVA WI T_5 $F_{7,32}$=7.25, P≤0.001). PRI and CTR2 represent stress factors, and in this case, these indexes may suggest that UC 67, AMF and two strains of bacteria treated plants had lower performances when compared to AMF+PSB1+PSB2+PSB3 (ANOVA PRI T_5

$F_{7,32}$=5.33, P≤0.001; CTR2 T_5 $F_{7,32}$=3.93, P≤0.01). This may suggest that, the treatment AMF and the 3 strains of bacteria, allowed plants to be more resilient under abiotic stress conditions that occurred before T_5, such as photosynthetic light efficiency, nutrient deficiency, low temperatures.

Two weeks after the start of the experiment, no differences were observed regarding NDVI, PRI and CTR2 (ANOVA NDVI T_2 $F_{7,32}$=0.79, P>0.05; PRI T_2 $F_{7,32}$=1.43, P>0.05; CTR2 T_2 $F_{7,32}$=1.29, P>0.05).

No significant difference was observed at final measures (one week before the end of the experiment) regarding most indexes which may be to absence of some of the abiotic stress or due pot effect (ANOVA SPAD T_9 $F_{7,32}$=0.89, P>0.05; WI T_9 $F_{7,32}$=2.06, P>0.05; NDVI T_9 $F_{7,32}$=1.31, P>0.05; CTR2 T_9 $F_{7,32}$=0.789, P>0.05). PRI showed that apart from AMF+PSB2, inoculated plants had higher values of PRI when compared to the UC 100 plants which may suggest that inoculants treated plants were under stress and continued to be more resilient to stress rather than the UC 100 (One-way ANOVA PRI 3 $_{F7,32}$=5.279, P≤0.001).

Table 3.2 Effect of six microbial consortia (one AMF species combined with different species of PSB) on SPAD and WI at five (T_5) and nine (T_9) weeks after the start of the experiment . For each column, different letters (mean ± SD, n=5) show significance at 5% level, according to Tuckey's HSD test.

Treatment	SPAD		WI	
	T5	T9	T5	T9
UC 100	28.9 ± 3.84 **ab**	45.9 ± 3.06	2.27 ± 0.01 **ab**	2.37 ± 0.01
UC 67	25.0 ± 3.81 **bc**	46.2 ± 2.30	2.27 ± 0.01 **a**	2.36 ± 0.02
AMF+PSB1	27.6 ± 5.45 **abc**	46.5 ± 4.31	2.25 ± 0.00 **c**	2.36 ± 0.01
AMF+PSB2	26.9 ± 3.90 **abc**	46.0 ± 3.12	2.25 ± 0.00 **c**	2.36 ± 0.01
AMF+PSB3	25.9 ± 1.33 **abc**	48.2 ± 4.68	2.25 ± 0.00 **c**	2.37 ± 0.01
AMF+PSB(1+2)	21.1 ± 1.63 **c**	45.5 ± 2.56	2.26 ± 0.01 **c**	2.37 ± 0.02
AMF+PSB(1+3)	24.5 ± 3.69 **bc**	48.7 ± 2.18	2.26 ± 0.00 **bc**	2.38 ± 0.01
AMF+PSB(1+2+3)	32.8 ± 3.35 **a**	44.0 ± 5.12	2.25 ± 0.00 **c**	2.38 ± 0.01

Table 3.3 Effect of six microbial consortia (one AMF species combined with different species of PSB) on SPAD and WI at five (T_5) and nine (T_9) weeks after the start of the experiment . For each column, different letters (mean ± SD, n=5) show significance at 5% level, according to Tuckey's HSD test.

Treatment	NDVI			PRI			CTR2		
	T2	T5	T9	T2	T5	T9	T2	T5	T9
UC 100	0.61 ± 0.03	0.52 ± 0.07 **ab**	0.60 ± 0.02	0.04 ± 0.02	0.04 ± 0.01 **ab**	0.05 ± 0.00 **c**	0.31 ± 0.03	0.37 ± 0.06 **ab**	0.28 ± 0.01
UC 67	0.61 ± 0.04	0.50 ± 0.07 **ab**	0.62 ± 0.04	0.05 ± 0.02	0.01 ± 0.03 **b**	0.06 ± 0.01 **bc**	0.32 ± 0.05	0.43 ± 0.08 **a**	0.27 ± 0.02
AMF+PSB1	0.59 ± 0.05	0.54 ± 0.06 **ab**	0.64 ± 0.01	0.03 ± 0.01	0.05 ± 0.02 **ab**	0.07 ± 0.00 **ab**	0.33 ± 0.05	0.38 ± 0.07 **ab**	0.26 ± 0.01
AMF+PSB2	0.61 ± 0.02	0.51 ± 0.03 **ab**	0.61 ± 0.04	0.04 ± 0.01	0.03 ± 0.03 **b**	0.07 ± 0.01 **abc**	0.30 ± 0.02	0.40 ± 0.06 **ab**	0.28 ± 0.03
AMF+PSB3	0.63 ± 0.03	0.53 ± 0.03 **ab**	0.61 ± 0.05	0.05 ± 0.01	0.05 ± 0.01 **ab**	0.06 ± 0.01 **ab**	0.29 ± 0.02	0.38 ± 0.03 **ab**	0.28 ± 0.03
AMF+PSB(1+2)	0.61 ± 0.02	0.47 ± 0.05 **b**	0.58 ± 0.03	0.03 ± 0.00	0.03 ± 0.01 **b**	0.07 ± 0.00 **ab**	0.32 ± 0.03	0.46 ± 0.07 **a**	0.29 ± 0.02
AMF+PSB(1+3)	0.61 ± 0.03	0.47 ± 0.05 **b**	0.63 ± 0.03	0.05 ± 0.02	0.03 ± 0.01 **b**	0.07 ± 0.01 **a**	0.31 ± 0.02	0.46 ± 0.05 **a**	0.26 ± 0.02
AMF+PSB(1+2+3)	0.59 ± 0.04	0.60 ± 0.03 **a**	0.60 ± 0.04	0.03 ± 0.02	0.07 ± 0.01 **a**	0.07 ± 0.00 **ab**	0.33 ± 0.05	0.30 0.03 **b**	0.29 ± 0.04

3.4. Discussion

AMF and PSB are widely distributed in soils. There is increasing evidence in literature that AMF and PSB work synergistically to provide benefits to each other and to the plant (Ordoñez et al. 2016; Zhang et al. 2016). The majority of the inoculants containing AMF and different species of bacteria increased plant performance (Table 3.1; Figure 3.2; Wu et al. 2005) based on morphological, physiological and P use efficiency traits of inoculated maize plants. However, results also call attention for the specificity of the interaction between the microbial community and the plant host.

3.4.1. Consortia and plant morphological characteristics

When plants were inoculated with AMF and a single species of PSB, a slight increment in plant's Dw (plant and shoot Dw) (Figure 3.2), height (Figure 3.3) and leaf area (Figure 3.4) was observed when compared to the UC 67 control. But, based on the same traits, those plants were not different from UC 100 treated plants. This is in line with what is often observed for biofertilizers application, there are tendencies but no significative trend is observed (Herrmann and Lesueur 2013; Owen et al. 2015). In our study the interaction between the AMF and each of the three PSB isolates was not negative, which may not always be the case (Ordoñez et al. 2016).

When plants were inoculated with AMF and two species of PSB no consistent plant response was observed. Plants inoculated with AMF+PSB(1+3) were similar to those inoculated with AMF and a single species of PSB. While, plant inoculation with the same AMF and another PSB (AMF+PSB(1+2)) resulted in a drastic decrease of plant performance, detected by a decrease of 40 % Dw (comprising shoot and root, Figure 3.2b), 57 % leaf area (Figure 3.4 and Appendix 6), and aproximately 60 % of root volume (Appendix 7) in comparison to UC 100 treated plants (Figure 3.2a). Results like those are rarely reported, because: 1) most of the studies related with biofertilizer design and efficiency test are performed with only one or two species (Owen et al. 2015); and 2) most of the studies aiming at assessing biofertilizer efficiency are performed under *in vitro* conditions or with sterilized soils (substrates) (Ordoñez et al. 2016).

The lower performance of plants inoculated with AMF+PSB(1+2) may be explained by the interaction of the inoculum species among them or with the soil microbial community. Studies suggest that the combination of inoculants does not necessarily produce an additive or synergistic effect. But may result in a competitive process among inoculants (Cavagnaro et al. 2006; Rydlová et al. 2011) or in a different response from the plant to each of the isolates of to the consortium as a unit (Berg et al. 2014).

Plants grown with UC 67 and inoculated with AMF and the three PSB species improved plant performance (about 5 % increase in plant Dw) when compared to the UC 100 plants (Figure 3.2). Studies suggest that PSB can stimulate root hairs and lateral roots elongation by producing indole-3-acetic acid (IAA), which provides more active sites and access for symbiotic associations with PSB and AMF (Aarab et al. 2015; Etesami et al. 2015; Ghorchiani et al. 2018). It is also known that plant growth improvement by microorganisms may be mediated through plant hormones (James et al. 2002). This might explain why AMF+PSB(1+2+3) treated plants were also taller (Figure 3.3), had higher leaf area (Figure 3.4), and more developed roots (Appendix 7FIGURE 3.3).

Taller plants with improved leaf and root areas are more fitted because they have improved access to light and nutrients (Falster and Westoby 2003).

3.4.2. Consortia and P nutrition and P efficiency

Previous studies, showed that the co-inoculation with AMF and PSB improved the efficiency of plants in acquiring P from low bioavailable sources, such as rock phosphate, and simultaneously enhances maize growth and yield when compared to a single microbial inoculation (Wahid et al. 2016; Ghorchiani et al. 2018).

Our results indicate that, depending on the combination of bacteria, there is a potential for positive interactions, such as cooperation, between AMF and PSB to promote P acquisition, however each consortium affected P use efficiency in distinct ways. Overall, no significant differences were observed among treatments regarding P content which could indicate that the differences in growth where due to the utilization of P acquired. In other words, different combination of microorganisms leads to different resource partition and allocation. It was also observed that plants treated with a combination of AMF and two species of bacteria had a tendency for higher shoot P concentration however one of these consortia (AMF+PSB(1+3)) was more efficient that the other (AMF+PSB(1+2)) in promoting plant growth and showed a tendency for overall higher P shoot content than the other. Besides this, treatments containing PSB3 showed a tendency for higher P content in the shoots and seemed to have the highest plant P content when compared with the other treatments however no significant differences in biomass were observed. It has been demonstrated that bacteria could behave differently in combination rather than in pure culture which may carry novel characteristic that affect plant growth (Roy et al. 2014). Biodiverse formulations that include more than one bacterial species, which represent a synthetic microbial community (Roy et al. 2014; Chiu et al. 2014), could be highly relevant for restoring ecological mechanisms once it is know that increasing species richness could increase ecological complexity (Schnitzer and Klironomos 2011).

Regarding P fertilizer recovery efficiency, plants treated with AMF+PSB(1+2+3) and AMF+PSB(1+3) were the most efficient in acquiring P applied to the soil in the form of RP, when compared to uninoculated control plants with full P fertilization (UC 100), having 21 % and 29 % more P in the plant tissues than UC 100 plants. Some studies demonstrated that a growing bacteria community produces and excretes metabolites that are not secreted by any member species when growing in isolation on that same medium (Roy et al. 2014). This distinct biosynthetic activity may be a result of a simple niche construction process, where the secretion or uptake of metabolites by one species modifies the composition of the environment and consequently modulates the metabolic activity of another species, causing it to produce and secrete metabolites it would not have produced if growing in isolation (Goebel et al. 2014). Furthermore, these could have implications on plant growth.

Thus, interactions among the different species of microorganisms could determine mechanisms that promote plant growth by the biofertilizer and the outcome for the host plant. It should be crucial to understand how biofertilizers would construct their environment and which specific metabolic capabilities they exhibit (Chiu et al. 2014).

3.4.3. Consortia and plant physiological response

Biofertilizers improve photosynthesis performance to confer plant tolerance to stress (Chi et al. 2010). Our results showed that apart from the consortium that had lower plant performance (AMF+PSB(1+2)), all the other treatments promoted high leaf chlorophyll content and high healthy leaf functions (SPAD and NDVI).

Plants treated with the most diverse consortium (AMF+PSB(1+2+3)) were more resilient to stress factors (detected with PRI and CTR2 indexes) when compared to the UC 67 and with the consortia with AMF and two species of bacteria. PRI is sensitive to the changes in carotenoid pigments (e.g. xanthophyll pigments) (Gamon et al. 1997). Carotenoid pigments are indicative of photosynthetic light use efficiency, or the rate of carbon dioxide uptake by foliage per unit energy absorbed (Thenot et al. 2002). CTR2 has also been reported to be sensitive to stresses in a wide variety of species (Carter 1994; Carter and Miller 1994). The stress agents include competition, herbicide, pathogen, ozone, mycorrhizae, island, senescence and dehydration (Carter 1994). This sensitivity is largely attributed to the "blue shift" from red to infrared light in the reflectance spectrum as chlorophyll concentration in plant tissue changes in response to stress (Carter et al. 1996). This may suggest that maize plants during the experimental time were unexpectedly under several stress besides the pre-established nutrient deficiency treatment that was overcome by the treatment AMF and the 3 species of bacteria that allowed plants to be more resilient.

Ethylene is important for normal development in plants as well for their response to stress (Morgan and Drew 1997). Its production is regulated by many factors such as temperature, light, nutrition and other plant hormones and its usually accelerated by environmental and biological stresses. Temperatures extremes, water stress, ultraviolet light and disease can cause stress symptoms in the plant and induce defence responses which could help to increase plant survival under adverse conditions (Glick 2005). Ethylene can function as an efficient plant growth regulator at very low concentrations (Abeles et al. 1992).

It is known that PGPR can contain the enzyme ACC (1-Aminocyclopropane-1-carboxylic acid) deaminase which can cleavage the plant ethylene precursor ACC. This can lower the levels of ethylene in the plant. Bacteria that have this trait, when bound to the roots of the plant can act as a sink for ACC which could protect stressed plants from some of the negative effects of stress ethylene (Glick 2005).

On the one hand some of the PSB could be acting as ACC sink, but on the other hand they could be giving the plant P and producing other compounds that could meet the needs of the plant and stimulate their growth and development, such as producing phytohormones, including auxins such as IAA or the production of antimicrobial metabolites such as antibiotics (Glick 2014). We believe that there should be a balance of these traits that promoted plant growth (Figure 3.9).

In the case of AMF and a single species of bacteria (PSB1, 2 or 3) it seemed that PSB3 could be reducing ethylene stress by producing ACC deaminase or by providing the plant P or other metabolites. While PSB1 and PSB2 they looked unable to avoid the stress of ethylene. When combining these two bacteria (PSB1+2) it seemed that the response of the plant to stress was higher as seen by the indices of stress and with the consequent lower plant performance when compared to the other treatments. This leads us to believe that the levels of stress overlap any nutritional benefits that this consortium could provide to the plant. On the other hand, it seems that the consortium with (PSB1+PSB3) has provided a boost in nutrients, hormones or other

metabolites to the plant that they overlapped with plant stress levels and allowed it to grow more in relation to the other consortium.

The consortium AMF+PSB(1+2+3) was in this case the most efficient in promoting plant growth. We believe that the combination of the different possible functions of each of the bacteria allowed for a boost of growth inducing metabolites, nutrients such as P, and the possible production of ACC deaminase, as well as, other compounds that allowed these plants to be more resilient to the stress factors to which they would be subjected to. In this context we hypothesize that PSB3 has potential for high ACC deaminase activity since it appears that stress levels are attenuated in its presence.

Further studies are needed to understand the mechanisms of action of these bacteria, in order to promote its use in agriculture to overcome biotic and abiotic stresses.

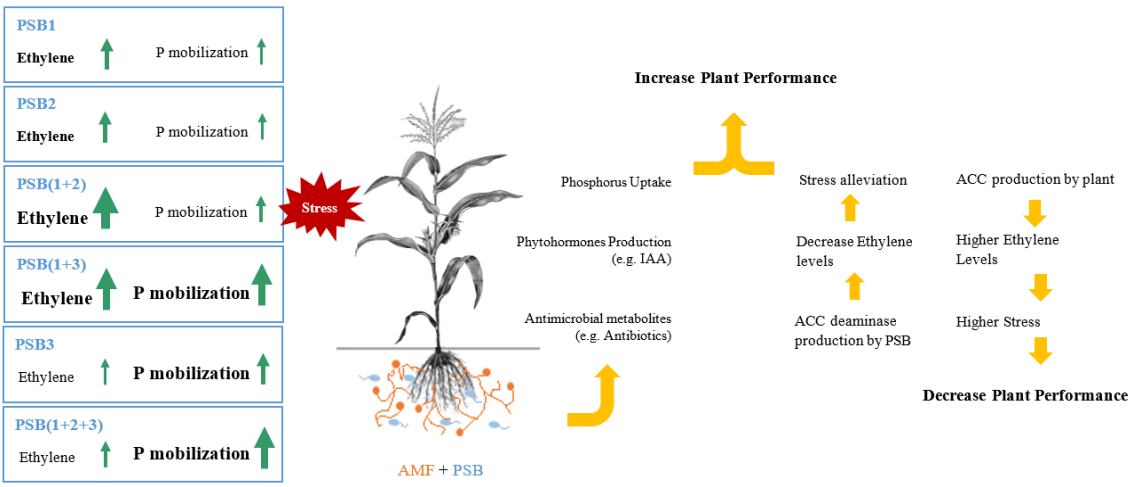

Figure 3.9 Schematic representation of possible mechanisms associated with the six microbial consortia (one AMF species combined with different species of PSB) on plant growth and stress resilience. We hypothesize that maize plants, during the experimental time, were unexpectedly under several other kinds of stress besides the pre-established nutrient deficiency treatment. On the left (blue rectangles) a representation of how we believe PSB may affect the plant's ethylene levels (stress can be regulated by ethylene levels) and P mobilization is depicted. Arrow size is proportional to an increase of ethylene or P mobilization in the plant, respectively. PSB(1+2) seemed to show the highest increase in stress levels, therefore decreasing plant performance. Some PSB can contain the enzyme ACC deaminase that can cleavage the plant ethylene precursor ACC. This can lower the levels of ethylene in the plant, which could protect stressed plants from some of the negative effects of stress ethylene. PSB could also be producing other compounds that could meet the needs of the plant and stimulate their growth and development, such as producing phytohormones, including auxins such as IAA, or the production of antibiotics.

We concluded that the combination between AMF and different PSB species may lead to synergistic consortia inoculants that can be used in agriculture to promote plant growth, P use efficiency and increase plant resilience to stress conditions. However, the fact that increasing bacterial species richness did not necessarily increase plant performance in unsterilized soil, highlights the need for understanding inoculants behaviour in presence of a microbial community. This kind of test, which is often omitted in such investigations, is essential to better understand and increase the knowledge in plant-microbial synergisms so that in the future it would be easier to predict the effects of a biofertilizer.

Chapter 4 – General discussion

The aim of this project was to understand if biofertilizers could act as an alternative management strategy to the current agricultural fertilization practices in order to reduce phosphorus (P) fertilizer application and increase plant P use efficiency in agricultural soils. Due to the relevance of the global P problematic such as P resource depletion and high P demanding agricultural practices (Cordell and White 2014), the use of biofertilizers is proposed as a novel strategy regarding P use efficiency.

In Chapter 2 it was demonstrated through a field experiment that microbial inoculants showed an extraordinary potential to resolve some of the current agricultural problems by enhancing agricultural productivity without increasing farmland area and promoting better P use efficiency by plants in agricultural soils. Furthermore, inoculants affected P allocation within the plant, resulting in lower P concentration in the grain mainly in phytate form. Since the grain is an edible part of maize plants and, no animal or human are able to digest and use this P form (which results in large quantities of P in excrements), this reduction of phytate would have environmental benefits avoiding eutrophication of rivers and lakes. As far as our literature research found, these latter results presented here, have not been demonstrated before using microbial inoculation.

Contrary to what was expected not all biofertilizers have increased productivity and P use efficiency. AMF inoculation in field trial did not promote plant growth, which could be explained by studies suggesting that mycorrhization is often negatively affected by high nutrient soil availability (Smith and Read 2008). From the two biofertilizers that increased crop productivity (AMF+PSB consortium and PSB alone), PSB was the most efficient in increasing nutritional value of maize (P content) which could be extremely useful in farms where the P availability in soil is low. It is known that PSB can increase shoot P content in a wide variety of crops (Manschadi et al. 2014; Kaur and Reddy 2015), like maize (Viruel et al. 2014; Zahid et al. 2015). However, increasing P content in the stover without altering P content in the grain has, once again, not been demonstrated before, using microbial inoculation. It would be relevant in future research to investigate to what extent these microorganisms could influence the expression of certain genes that have already been described as influencing the partition of P in cereals (Yamaji et al. 2017) and whether this change is related to the production of certain compounds such as phytohormones.

When looking at the European panorama, where soils contain more P than recommended, even if unavailable to plants (MacDonald et al. 2011; Amery, F., Schoumans 2014), the results obtained with the consortium inoculum are more relevant. AMF+PSB consortium was the most efficient in promoting plant growth per unit of P in the tissue. These traits have been demonstrated by studies based on the selection of plant traits (breeding programs) (Rose et al. 2013; Pariasca-Tanaka et al. 2015; van de Wiel et al. 2016; Vandamme et al. 2016) or mutants (Lin et al. 2005; Zhao et al. 2008; Yamaji et al. 2017). Therefore, the results obtained in this experiment may be a step towards more research in this field with the aim of increasing P utilization efficiency (PUE). This consortium was further tested on Chapter 3 as a way to reduce P inputs, ideally by 33 %, as recommended by the EU, to see if plant performance was maintained. This aligns with the latest proposed objective for European agriculture which is the sustainable intensification of agriculture

intended to strengthen food production with minimal negative environmental impacts and zero increase in land degradation (European Commission 2018)

In Chapter 3, through a pot experiment, under greenhouse condition, poor P availability conditions were potentiated (33 % fertilization reduction) in order to evaluate whether different microbial consortia species richness and composition differentially affect plant performance. The PSB bacteria diversity (species richness and composition) of the tested consortia varied by adding more two species of PSB to the species 1 (PSB1), used in Chapter 2, species 2 (PSB2) and species 3 (PSB3), and maintained the same AMF species (also used in Chapter 2). However contrary to what was expected, increasing inoculum bacterial species richness did not improved plant performance in all cases.

In Chapter 3, it was observed that consortia with similar species richness, that included one AMF species combined with one single species of PSB (1, 2 or 3), showed similar plant growth, physiological and P nutrition characteristics among them however no significant different from the uninoculated controls. However, when rising species richness in consortia by combining AMF with two species of bacteria there was inconsistent effects in plant performance depending on the consortium. One of the consortia (AMF+PSB(1+3)) increased plant growth and P nutrition, while the other (AMF+PSB(1+2)) did not promoted these traits when compared to the control plants. Nevertheless, plants treated with these two consortia consistently showed to be less resilient to abiotic stress factors (similar to the uninoculated control with P fertilization reduction), assessed with CTR2 and PRI physiological indexes when compared to a consortium with one species of bacteria. A possible explanation for this inconsistency in plant performance and stress alleviation by the two consortia with the same species richness (2 bacteria species) comparatively to the consortia with the same single bacteria species, is based in some studies demonstrating that a microbial community, where all the microorganisms were growing together produces and secretes metabolites that are not secreted by any member species when growing in isolation (Chiu et al. 2014). These biosynthetic activity differences could be a result of a simple niche construction process (Schnitzer and Klironomos 2011; Goebel et al. 2014), where the secretion or uptake of metabolites by one species modifies the composition of the environment and consequently modulates the metabolic activity of another species, causing it to produce and secrete metabolites it would not have produced if growing in isolation (Chiu et al. 2014). Furthermore, these microbial metabolic differences could have implications on plant growth, and plant stress tolerance.

Plants treated with the most diverse consortium here tested, resulted of combining AMF and three species of bacteria (AMF+PSB(1+2+3)) had the best performance, revealing growth morphology, P nutrition, P acquisition efficiency similar to an uninoculated control with 100 % P fertilization. Thus, the highest P use efficiency for agricultural soils was achieved with the most diverse consortium. This could be a result of niche complementarity where there is an increase in resource use efficiency (Schnitzer and Klironomos 2011; Goebel et al. 2014) which is known to enhance ecosystem functions mediated by microorganisms (Goebel et al. 2014). Thus, interactions among the different species of microorganisms could determine mechanisms that promote plant growth by the biofertilizer and the outcome for the host plant. It should be crucial to understand how biofertilizers would construct their environment and which specific metabolic capabilities they exhibit (Chiu et al. 2014).

From Chapter 2 and Chapter 3 it can be concluded that a microbial consortium containing AMF+PSB can improve plant performance and P use efficiency in agricultural soils. Our results

also indicate that, depending on the bacterial combination, there is a potential for positive interactions, such as cooperation, between AMF and PSB to promote P acquisition, but each consortium affected the P use efficiency in different ways. Our results point out the need to take into account the relationships between the components of the consortium and between the host crop and the biofertilizer, which may explain the inconsistencies reported for the biofertilizer effect.

Despite the extensive number of studies and findings in literature about the beneficial use of microorganisms on plant growth and nutrient acquisition (Vessey 2003; Antoun 2012; Ordoñez et al. 2016; Zhang et al. 2016), the application of biofertilizers based on microbial inoculants in agricultural practices is still hampered by large variability of abiotic and biotic factors (Owen et al. 2015; Rodriguez and Sanders 2015).

The existence of microorganisms that can promote plant growth, or which promote the availability and acquisition of nutrients, has been known for many years (Richardson et al. 2009a). Many authors consider biofertilizers management an option potentially useful but in the meantime, it has not yet been taken the step of using these microorganisms as a reliable and effective measure (Alori et al. 2017). This might be because of contradictory results from system to system. Nowadays, agriculture is based on the use of mineral fertilizers because they are easily accessible and the predictability of yields based on its application is very high and it is standardized for a wide range of crops and soils, however is likely to cause negative impact in respects to both environment and economy (Tiessen 2008; Childers et al. 2011; Reijnders 2014). It should be emphasized that biofertilizers should not be seen as just the replacement of mineral fertilizers, but a complementary tool used in agriculture allowing the reduction of those for a more sustainable agriculture.

More studies that evaluate the effectiveness of biofertilizers in field and in pot using nonsterile field soil are needed so that in the future it is possible to gather all the results obtained (e.g. perform a meta-analysis) and understand which the best strategy is to increase the predictive power of biofertilizers. It is crucial to realize how as a given formulation of biofertilizer will "behave" depending on the abiotic and biotic characteristics of the target environment. These are the key regulators of microbial ecology and likely alter the persistence and efficacy of biofertilizers in the field (Herrmann and Lesueur 2013). Despite the literature describing the unpredictability of the biofertilizers (Herrmann and Lesueur 2013; Owen et al. 2015), in this work the inoculant (AMF+PSB1) proved some consistency in the two tested experimental systems with low and high P availability; always promoted plant growth comparatively to the control plants at the same level of fertilization. In the field system with high P availability, this consortium performs significantly better than the control treatments, in pot system with low P availability this difference was not significant but there was a trend to improve growth comparatively to control plants.

Ideally, the "perfect" formulation of biofertilizers should be adapted to different climate conditions such as temperature and precipitation, as well as, should be designed to act in different soil types, fertility and management. These conditions can shape the soil environment and patterns of microbial biogeography (Pasternak et al. 2013) thus, affecting microbial activity and community composition (Francioli et al. 2016).

There is still a lot of work ahead, not only regarding the formulation of a "super" biofertilizer but also making it economically feasible. The higher the number of microorganisms present in a

product, the more resources a company will have to spend in order to produce said product (Owen et al. 2015). But in the medium to long term can this be the most economically and environmentally cost-effective solution? Because it would allow a reduction in the mineral fertilizer inputs, some costs associated with the acquisition of mineral fertilizers could be invested in biofertilizer, allowing EU to be more auto-sufficient in terms of agriculture and depending less and less on countries holding the monopoly of rock phosphate, which are the ones that define its prices.

It is necessary to find more effective and economic measures, not only using a single method but a rational combination of those that result more efficiently depending on the crop, geographical location, excess or scarcity of soil P, in order to develop an integrated and conscious application program for certain biofertilizers (Owen et al. 2015; Malusà, E., F. Pinzari 2016). To ensure the future, it is necessary to develop more sustainable agricultural systems. It is crucial to understand the impact of our agricultural practices and how integrated management could contribute to a sustainable intensification, enabling us to achieve the current global challenge of providing sufficient and nutritional food to the entire world population without increasing our area agricultural (Tilman et al. 2002). This change in management could contribute substantially to the conservation of natural resources and result in a considerable reduction in environmental pollution (Childers et al. 2011; Reijnders 2014). It is important to improve the sustainability of production systems and to increase biodiversity. Perhaps its most important role will be in the development of tools in which it will be possible to reconcile agricultural food production with sustainable methods of crop protection while maintaining biodiversity (Owen et al. 2015).

The Common Agricultural Policy (CAP) in the EU could promote the use of biofertilizers as a tool to correct the contribution of agriculture to the imbalance of the biogeochemical cycle of P. In this way, the EU countries could become less dependent on the availability of phosphorite stocks, as well as achieving a sustainable intensification of agriculture. The use of rhizospheric microorganisms such as AMF and PSB in polymicrobial multifunctional consortia may be the wake-up call for a more sustainable agricultural intensification based on a sustainable P-use strategy in agricultural soils.

This work contributes to a growing body of scientific evidence supporting alternative methods of achieving a more sustainable agriculture. By sharing the knowledge acquired with the scientific community, farmers and policy makers throughout the various European countries, local agricultural practices and policies could be considered in order to select those that are most compatible with soil conservation, as they are increasingly being pressured by climate change and population growth.

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Appendices

Appendix 1 - Summary of the meteorological and agrometeorological conditions of the Lisbon district from June to September 2016

Table A – Lisbon district meteorological elements: minimum and maximum temperature (ºC), precipitation (mm), relative humidity (%) and wind (km/h). Values represent a mean of 10 days and were collected at 9UTC. Temperature and precipitation were registered at 1.5m high and relative humidity and wind at 10m high (Adapted from IPMA, 2016).

Lisbon station	**Minimum temperature (ºC)**			**Maximum temperature (ºC)**			**Precipitation (mm)**			**Relative humidity (%)**			**Wind (Km/h)**		
Month third	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
June	16.1	15.9	17.3	26.4	24.1	29.1	0.0	1.4	0.0	63.0	66.5	66.2	11.5	15.2	14.9
July	17.3	18.9	19.5	29.6	31.5	31.4	0.0	0.0	0.0	67.7	53.2	62.5	13.4	14.3	13.8
August	19.3	19.0	18.0	31.7	31.3	30.9	0.0	0.0	0.0	59.7	59.1	66.3	14.3	12.7	12.4
September	19.6	16.6	16.5	31.9	27.4	27.2	0.0	14.0	0.0	64.8	65.8	66.7	11.8	12.8	12.8

Table B – Lisbon district agrometeorological elements: soil temperature at 5cm and 10cm depth (ºC), reference evapotranspiration (mm) and soil water content (%). Values represent a mean of 10 days. Reference evapotranspiration (from 00UTC to 24UTC) was estimated with "ALADIN" numerical model and according to the FAO method for each third of a month and the accumulated value in the current hydrological year (1st October through 30th September), (Adapted from IPMA, 2016).

Lisbon station	**Soil temperature 5cm (ºC)**			**Soil temperature 10cm (ºC)**			**Reference evapotranspiration (mm)**				**Soil water content (%)**
Month third	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	Accumulated	Until the end of the month
June	21.7	22.7	24.9	21.3	22.5	24.7	50.5	53.1	58.7	705.3	72.0
July	26.2	27.5	28.3	25.9	27.1	28.1	55.2	63.3	64.4	888.3	51.2
August	28.2	28.1	26.8	28.0	28.1	26.9	61.3	53.0	56.6	1059.1	28.6
September	27.2	23.0	22.3	27.3	23.4	22.6	49.7	40.3	40.0	1189.0	18.7

Appendix 2 - Isolation and counting protocol

AMF spores were isolated from 10 g of commercial product by wet sieving through 63 and 20 µm sieves. The harvested were well suspended in 15 mL of distilled water in a 50 mL Falcon tube. A 30 mL sucrose solution (70 % v/w) was injected into the bottom of the tube, forming a stepped density gradient that was centrifuged at 3000 rpm for 2 minutes. Spores of AMF were collected from the interface of the sucrose solution, washed with tap water on a 63 and 20 µm sieves for 2 minutes, and transferred to Petri dishes. Spores were counted in three replications under stereomicroscope at 100× magnification. Spore abundance was expressed as the number of AMF spores per gram of subtract.

Appendix 3 – Photos of the effect of three inoculants (AMF, PSB, AMF+ PSB) on maize growth (plant height, number of tillers, number of ears, size of ears) under field conditions. Photos were taken 22 of September 2016

Control

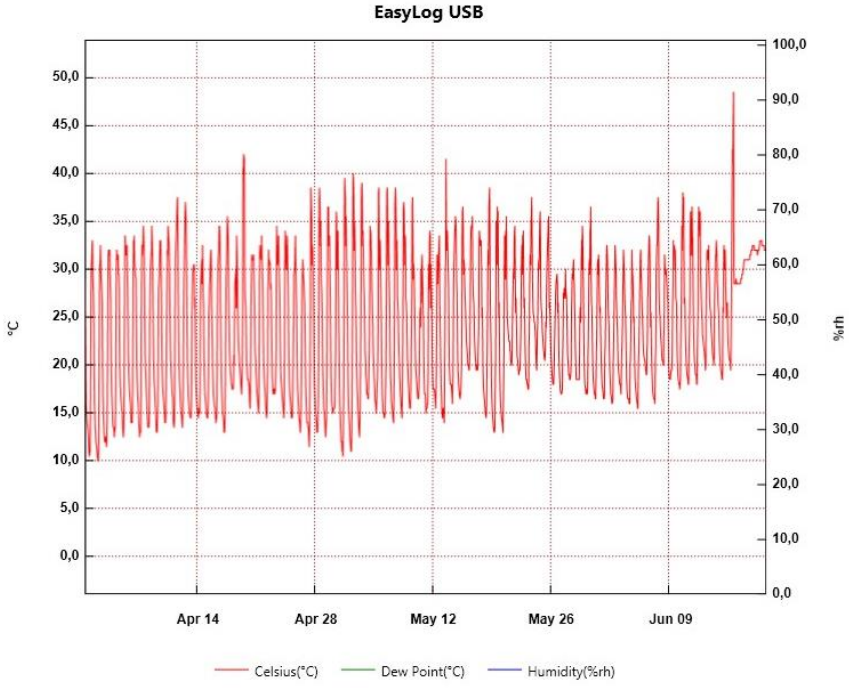
AMF

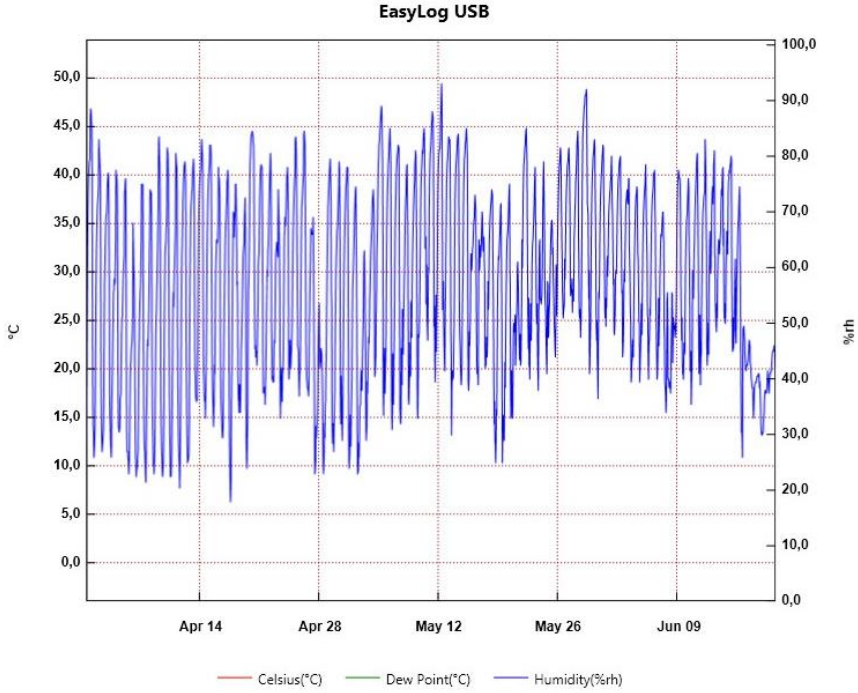
PSB

AMF+ PSB

Appendix 4 – Daily average temperature and humidity in the greenhouse experiment

Temperature (ºC) is represented in red and humidity (%) in blue. Data was collected inside the greenhouse every two hours a day with EasyLog USB.





Appendix 5 – Script to calculate leaf area with ImageJ (FIJI)

Set scale

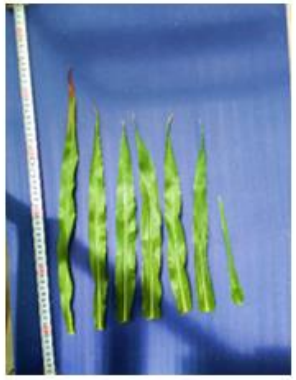
Data was calculated using the following script

```
// input = folder where the images are
e.g. input = "C:\\Users\\Inês\\Desktop\\Area_Foliar\\";
// output = folder where the treated images will be sent to (the folder must exist)
output = "C:\\Users\\Inês\\Desktop\\test_macro\\";

function area_selection(input, output, filename) {
	open(input + filename);

	// Color Thresholder 2.0.0-rc-61/1.51n
	// Autogenerated macro, single images only!
	min=newArray(3);
	max=newArray(3);
	filter=newArray(3);
	a=getTitle();
	run("HSB Stack");
	run("Convert Stack to Images");
	selectWindow("Hue");
	rename("0");
	selectWindow("Saturation");
	rename("1");
	selectWindow("Brightness");
	rename("2");
	min[0]=0;
	max[0]=89;
	filter[0]="pass";
	min[1]=36;
	max[1]=255;
	filter[1]="pass";
	min[2]=50;
	max[2]=255;
	filter[2]="pass";
	for (i=0;i<3;i++){
```

```
  selectWindow(""+i);
  setThreshold(min[i], max[i]);
  run("Convert to Mask");
  if (filter[i]=="stop")  run("Invert");
 }
 imageCalculator("AND create", "0","1");
 imageCalculator("AND create", "Result of 0","2");
 for (i=0;i<3;i++){
  selectWindow(""+i);
  close();
 }
 selectWindow("Result of 0");
 close();
 selectWindow("Result of Result of 0");
 rename(a);
 // Colour Thresholding----------

 saveAs("Jpeg", output + filename);

 run("Analyze Particles...", "  show=Masks clear summarize");
 close();
 close();
}

setBatchMode(true);  // na net diz que isto é preciso...

list = getFileList(input);
for (i = 0; i < list.length; i++)
        area_selection(input, output, list[i]);

setBatchMode(false);
```

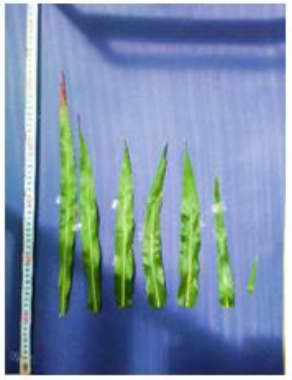
Appendix 6 – Photos of the effect of different inoculants of microbial consortia on leaf area. . Photos were taken at T_{10}, 2017

UC 100

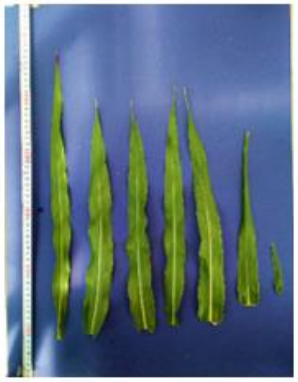


UC 67

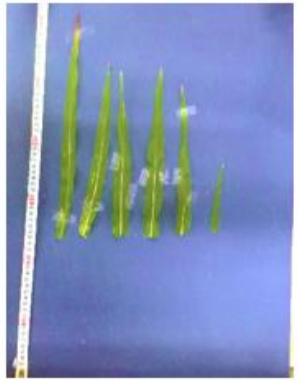
AMF+PSB1



AMF+PSB2



AMF+PSB3



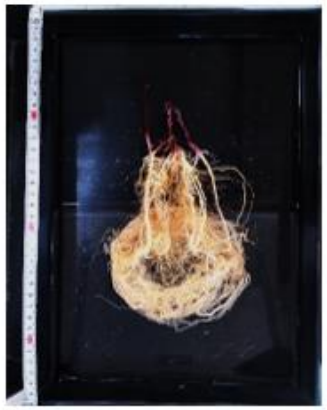
AMF+PSB(1+2)

AMF+PSB(1+3)

AMF+PSB(1+2+3)

Appendix 7 – Photos of the effect of different inoculants of microbial consortia on root morphology. Photos were taken at T_{10}, 2017

UC 100



UC 67

AMF+PSB1

AMF+PSB2

AMF+PSB3

AMF+PSB(1+2)

AMF+PSB(1+3)

AMF+PSB(1+2+3)

Appendix 8 – Photos of the effect of different inoculants of microbial consortia on maize growth. Photos were taken at T_8, 2017

UC 100

UC 67

AMF+PSB1

AMF+PSB2

AMF+PSB3

AMF+PSB(1+2)

AMF+PSB(1+3)

AMF+PSB(1+2+3)